Biochemical and molecular analyses of the biosynthesis pathway of indole derivatives in *Piriformospora indica*

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Declaration

I hereby declare that the dissertation entitled "Biochemical and molecular analyses of the biosynthesis pathway of indole derivatives in *Piriformospora indica*" submitted to the Department of Biology, Philipps-Universität Marburg, is the original and independent work carried out by me under the guidance of the PhD supervisors, and the dissertation is not formed previously on the basis of any award of Degree, Diploma or other similar titles.

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(Date and Place) (Magdalena Hilbert)

Parts of this work were published in the following articles:

Zuccaro A., Basiewicz M., Zurawska M., Biedenkopf D., Kogel K.H. 2009 Karyotype analysis, genome organization, and stable genetic transformation of the root colonizing fungus *Piriformospora indica*. Fungal Genet Biol.; 46(8):543-50.

Hilbert M., Voll L., Ding Y., Hofmann, J., Sharma M., Zuccaro A. 2012 Indole derivative production by the root endophyte *Piriformospora indica* is not required for growth promotion but for biotrophic colonization of barley roots. New Phytologist, 196(2):520-34.

“The highest reward for a person’s toil is not what they get for it, but what they become by it.”

— John Ruskin (1819-1900)

Moim Rodzicom i Mężowi

Summary

The mutualistic root endophyte *Piriformospora indica* has the ability to colonize a wide range of plants including the monocot barley (*Hordeum vulgare*) and the dicot model plant *Arabidopsis thaliana*. The colonization of both, Arabidopsis and barley is characterized by a biphasic colonization strategy with an initial biotrophic interaction followed by a cell death associated phase. During both phases fungal inter- and intracellular growth is restricted to the root cortex cells and can never be observed in the endodermis or the central cylinder. The colonization of root cells by *P. indica* is often associated with beneficial effects to the host, such as growth promotion and changes in root morphology. The broad host range and the widely conferred benefits to the hosts suggest that the beneficial outcome could be based on general mechanisms and signaling pathways common to many different plant species. One such mechanism could be the recruitment of phytohormone pathways by *P. indica*. Beside their function in plant developmental processes, phytohormones were recently described to be involved in plant defence responses. Secretion of indole-3-acetic acid (IAA) by *P. indica* into the growth medium has already been reported. In this PhD study a tryptophan dependent IAA production pathway was identified in *P. indica* using biochemical and molecular methods. The main goals were focused on the functional analyses of the *P. indica* tryptophan dependent IAA pathway and how this may affect compatibility during the biotrophic interaction between *P. indica* and barley roots. For this, suitable molecular tools such as a PEG-mediated genetic transformation, a GFP reporter and an RNAi-mediated silencing system were established or optimized for use in *P. indica*. Time course transcriptional analyses after exposure to tryptophan designated the tryptophan aminotransferase *piTam*1 gene as the top candidate gene involved in the production of IAA in *P. indica*. A green fluorescence protein (GFP) reporter study and transcriptional analysis of colonized barley roots showed that *piTam*1 is induced during the biotrophic phase. Via an RNAi-mediated gene silencing *piTam*1 was identified as a key gene involved in the first step of auxin biosynthesis. RNAi transformants impaired in auxin production were characterized by a less compact colony growth phenotype and differences in their ability to utilize indole-3-acetaldehyde

(IAD). Additionally, silencing of the *piTam*1 gene resulted in a reduced *P. indica* colonization of barley roots at 3 days after inoculation (dai) but the elicitation of growth promotion was not affected compared with barley colonized by the *P. indica* wild-type strain. Consistently an increased amount of free IAA and free indole-3-lactate (ILA), a byproduct of *P. indica* IAA biosynthesis pathway, could be detected in *P. indica* colonized barley roots compared to non colonized control plants at this time point Given the large amount of IAA detected *in planta* at 3 dai and the amount of IAA produced by *P. indica* in culture after tryptophan induction, it is unlikely that the differences in free IAA levels observed *in planta* are merely derived from fungal IAA. This suggests a local accumulation of both fungal and plant IAA during the initial biotrophic phase and is consistent with changes in IAA signaling and biosynthesis observed in barley transcriptome at this time point.

Zusammenfassung

Der mutualistische Wurzelendophyt *Piriformospora indica* besitzt die Fähigkeit eine Vielzahl unterschiedlicher Pflanzen, einschließlich der monokotylen Gerste (*Hordeum vulgare*) und der dikotylen Modelpflanze Ackerschmalwand (*Arabidopsis thaliana*), zu besiedeln. Die Besiedelung sowohl von Ackerschmalwand als auch von Gerste ist durch eine Besiedlungsstrategie charakterisiert, welche sich in zwei Phasen unterteilt: einer initialen, biotrophen Interaktion und einer, sich daran anschließenden, Zelltot-assoziierten Phase. Im Verlauf beider Phasen können Pilzhyphen inter- und intrazellulär beobachtet werden. Das Pilzwachstum beschränkt sich hierbei jedoch auf die Cortexzellen der Wurzel, eine Besiedelung von Endodermis oder Zentralzylinder konnte nie beobachtet werden. Die Besiedlung der Wurzelzellen durch *P. indica* ist dabei oft mit vorteilhaften Effekten für den Wirt assoziiert, wie zum Beispiel Wachstumsförderung und Änderungen der Wurzelmorphologie. Das breite Wirtsspektrum und die positiven Effekte für den Wirt lassen auf eine generelle Interaktionsstrategie schließen, bei der Proteine des Pilzes mit pflanzlichen Signalwegen interagieren, die in vielen verschiedenen Pflanzenarten vorkommen. Ein solcher Mechanismus könnte der Eingriff von *P. indica* in die Phytohormonbiosynthese sein. Hierzu konnte kürzlich gezeigt werden, dass Phytohormone neben ihrer Rolle als Regulatoren von Entwicklungsprozessen in Pflanzen auch an der Pflanzenabwehr beteiligt sind. Des weiteren wurde bereits die Sekretion von Indol-3-Essigsäure (IAA) in das Wachstumsmedium durch *P. indica* beschrieben. In der vorliegenden Doktorarbeit wurde die Produktion von IAA aus Tryptophan in *P. indica* durch eine Reihe biochemischer und molekularer Methoden charakterisiert. Die Hauptziele lagen dabei auf einer funktionellen Untersuchungen der IAA Biosynthese in *P. indica* und welche Auswirkung diese auf die Kompatibilität während der biotrophen Interaktion von *P. indica* mit Gerstenwurzeln haben. Hierfür wurden geeignete molekulare Methoden für den Gebrauch in *P. indica* etabliert und optimiert. Zu diesen gehören eine PEG-vermittelte genetische Transformation, sowie ein GFP-Reporter- und ein RNA-Interferenz (RNAi) System. Mittels zeitabhängiger Analyse der Transkriptionsrate nach Zugabe von Tryptophan, konnte das Tryptophan-Aminotransferase Gen *piTam*1 als

Kandidat für die Beteiligung an der IAA-Produktion in *P. indica* identifiziert werden. Die Untersuchung des *piTam*1 Promoters durch ein GFP-Reporter System sowie transkriptionelle Analysen des Genes in besiedelten Gerstenwurzeln, verifizierten die Aktivierung der *piTam*1 Expression während der biotrophen Phase. Durch Suppression dieser Expression mittels RNA-Interferenz (RNAi) wurde *piTam*1 als Schlüsselgen in der Umwandlung von Tryptophan in Indol-3-Pyruvat (IPA) identifiziert, welches den ersten Schritt in der Produktion von Auxin aus Tryptophan darstellt. RNAi Transformanden, deren Auxin-Produktion gehemmt war, zeigten ein weniger kompaktes Koloniewachstum und unterschieden sich in ihrer Fähigkeit Indol-3-Acetaldehyd (IAD) umzusetzen. Zusätzlich führte die gehemmte IAA-Biosynthese zu einer verminderten Besiedlung von Gerstenwurzeln durch *P. indica* während der ersten drei Tage der Interaktion, wobei jedoch die wachstumsfördernden Effekte nicht beeinflusst wurden. Konsequent, ein erhöhter Gehalt an freiem IAA und Indol-3-Lactat (ILA) einem Nebenprodukt der IAA Biosynthese in *P. indica*, konnten in besiedelten Wurzeln im Vergleich zu Kontrollwurzeln beobachtet werden. Obwohl 3 Tage nach Inokulation mit *P. indica* eine erhöhte IAA Konzentration *in planta* gemessen wurde, ist es unwahrscheinlich, dass die beobachteten Unterschiede lediglich durch pilzliches IAA verursacht wurden. Dies lässt eine Akkumulation von pilzlichen und pflanzlichen IAA vermuten und stimmt mit den Änderungen der IAA Signalübertragung und der Biosynthese im Gerste-Transkriptom zu diesem Zeitpunkt überein.

Abbreviations

AAT – aromatic-amino-acid-transaminase

ABA - abscisic acid

BLASTp – protein-protein Basic Local Alignment Search Tool (Altschul et al.,1990)

bp – base pair

cDNA - complementary DNA

CM – complete medium

CTAB - cetriumonium bromide

dai – days after inoculation

DEPC - diethylpyrocarbonate

DIG - digoxigenin

DMSO - dimethyl sulfoxide

DNA - desoxyribonucleic acid

DNase - desoxyribonuclease

dNTP - desoxyribonucleoside triphosphate

ET - ethylene

EtOH - ethanol

FW – fresh weight

GA - gibberellins

GFP – green fluorescence protein

GPD - glyceraldehyde-3-phosphate dehydrogenase

hai – hours after inoculation

Hpt – hygromycin phosphotransferase gene

IAA – indole-3-acetic acid

IAD – indole-3-acetaldehyde

IAM – indole-3-acetamide

IAN – indole-3-acetonitrile

ILA – indole-3-lactic acid

IPA – indole-3-pyruvate

JA – jasmonic acid

LC-MS/MS - Liquid Chromatography Tandem Mass Spectrometry

MM – minimal medium

MS - medium Murashige & Skoog medium

PCR – polymerase chain reaction

PEG – polyethylene glycol

qPCR – quantitative real-time PCR

Rf - retention factor

RNA - ribonucleic acid

RNase A - ribonuclease A

RT - room temperature

SA – salicylic acid

SAR - systemic acquired resistance

TAM – tryptamine

TEF - translation elongation factor 1-α

TLC – Thin Layer Chormatography

TOL – tryptophol

TRP – tryptophan

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1. Introduction

1.1. Phytohormones

The term hormone comes from the Greek word "hormon" which means "set in motion". In medicine it is used to describe molecules which are synthesised by the organism itself, secreted by specialized cells and affect cells in other parts of the organism. These chemical signals can alter the cell metabolism in very small amounts. Plants have also generated their own endogenous growth regulators which act adequate to the growth stage and respond to the unexpected environmental stresses. Because the function of these small molecules, though differing in chemical structure, resembles that of animal hormones, these plant growth regulators are named phytohormones (Letham, 1969; Davies, 2010). Despite some debate regarding which plant growth regulators entirely fulfil the hormone definition, five principal types have been recognized: auxin, cytokinins (CK), gibberellins (GA), ethylene (ET) and abscisic acid (ABA). Some additional molecules like salicylic acid (SA), jasmonic acid (JA) or brassinosteroids (BR) are often classified as phytohormones because of their important role in plant growth, development and defence processes, even though they act mainly locally (Crozier et al. 2000). Recently a new class of terpenoid lactones, strigolactones, was accepted within the phytohormone category because of their ability to inhibit plant shoot branching (Umehara et al., 2008; Vogel et al., 2010). These carotenoid-derived hormones can stimulate the branching and growth of symbiotic arbuscular mycorrhizal fungi helping in the recognition, contact and establishment of the symbiotic association (Akiyama et al., 2005).

1.2. Plant defence responses and the role of phytohormones

In order to survive in an environment replete with different kinds of microorganisms, plants have developed complex multiple protective mechanisms. The first lines of plant defence are physical barriers and the production of toxic secondary metabolites (Osbourn et al., 1996). Nevertheless, successful microbes are able to overcome these obstacles and penetrate into the plant cells. During penetration attempts by the microorganism, pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) like

lipopolysaccharides (LPS), peptidoglycan (PGN), flaggelin, bacterial elongation factor Ef-Tu, β-glucan, chitin or chitosan are released (Zeidler et al., 2004). They can be recognized by pattern-recognition receptors (PRRs) localized on the plant cell surface (Zipfel, 2008). Identification of MAMPs results in the activation of pattern-triggered immunity (PTI) which can be subsequently suppressed by microbial secreted effectors (Göhre & Robatzek, 2008). Nucleotide-binding domain leucine-rich repeat proteins (NB-LRR) play a major role in binding of pathogen effectors and inducing effector-triggered immunity (ETI), which can result in a hypersensitive response (HR, Jones & Dangl, 2006). Recognition of MAMPs also correlates with the activation of long-lasting systemic immunity, referred to as systemic acquired resistance (SAR). The establishment of SAR is mainly influenced by salicylic acid (SA). Biosynthesis of this phytohormone drastically increases upon pathogen penetration (Halim et al., 2007). Accumulation of SA in the cell results in redox changes, which leads to the dissociation of NPR1 (nonexpressor of pathogenesis related (PR) genes1) multimers and transport of active monomers from the cytoplasm into the nucleus (Després et al., 2000). NPR1 is known to be a cofactor for transcription factors. Degradation of NPR1 acts as a molecular switch (Fu et al., 2012). NPR1 binds to the basic leucine zipper transcription factors, thereby stabilizing their binding to PR promoter sequences (Després et al., 2000). This physical interaction positively modulates the SA-dependent expression of *pathogenesis related* (PR) genes like PR-1, PR-2, PR-5, which encode antimicrobial proteins (Thomma et al., 2001). Therefore, accumulation of SA or its exogenous application correlates with enhanced resistance to biotrophic and hemi-biotrophic pathogens. Recently, Wu et al. (2012) proposed that SA binds to NPR1 through two cysteine residues ($Cys^{521/529}$) in presence of copper ions. Fu et al. (2012) expanded the knowledge of the correlation between NPR1 and SA but in contrast to Wu et al. (2012), they have shown that SA itself did not bind to NPR1 but to its paralogues NPR3 and NPR4. These proteins control the degradation of NPR1 by CUL3 E3 ligase (Cullin3 ubiquitin E3 ligase; Fu et al., 2012) depending on SA concentration. Basal resistance in the cell corresponds with a low SA concentration which leads to the balanced interchange between free NPR1 which play role in defence gene activation and NPR1 bound to NPR4 which as a complex is degraded in the proteasomes. Upon pathogen attack, SA levels increase significantly both locally and systemically. Inside the lesion

high SA levels lead to CUL3NPR3-mediated degradation of NPR1. This upregulates the PR genes and results in activation of effector-triggered programmed cell death. Systemically, the lower level of SA limits NPR1-NPR3 interaction, enabling NPR1 to accumulate. Accumulation of NPR1 in neighbouring cell inhibits programmed cell death and establishes SAR (Fu et al., 2012).

An increasing number of studies have indicated that classical phytohormones are not only plant growth regulators but also important determinants of plant immunity and that their cooperation with SA and JA plays a significant role in supplying an adequate defence response (Pieterse et al., 2009). Fig. 1 represents a simplified summary of the influence of well-characterized phytohormones on the establishment of susceptibility against different pathogens.

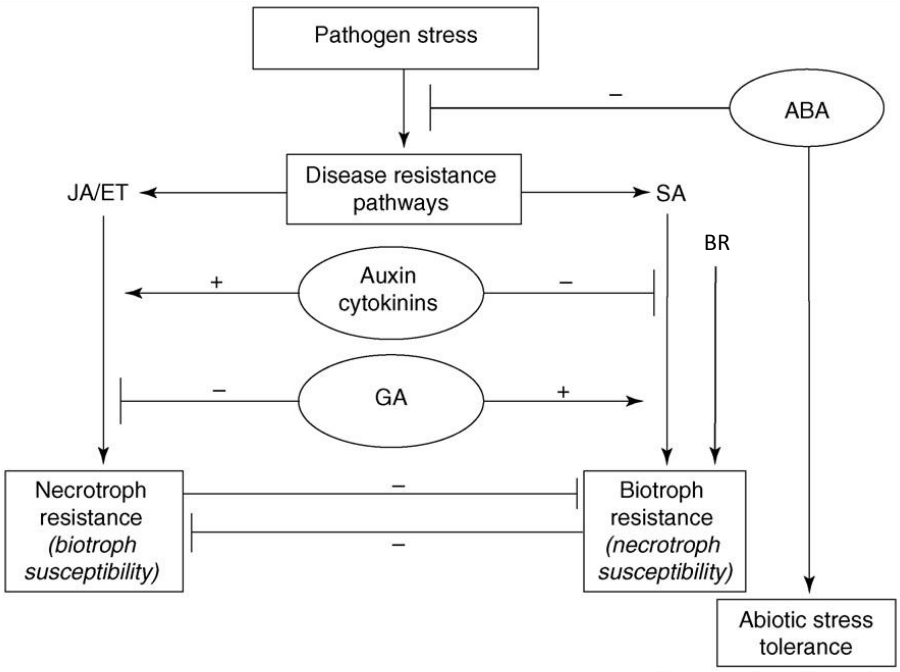

Fig. 1 Scheme representing a simplified model of the role of phytohormones in the establishment of susceptibility or resistance to microorganisms with different life styles (Robert-Seilaniantz et al., 2007). ABA: abscisic acid, JA: jasmonic acid, ET: ethylene, SA: salicylic acid, GA: gibberellic acid, BR: brassinosteroids.

Microbial induction of SA biosynthesis and signaling in the host facilitates necrotrophic penetration and decreases efficiency of biotrophic infections. Similar effects are observed by altering GA and BR signaling. Increased signaling of JA, ET, auxin and/or CK makes plants more susceptible to invasions of biotrophs and more resistant to necrotrophs. Signaling crosstalk between some phytohormones has also been observed.

Spoel et al. (2003) showed that activation of SAR by enhanced SA signaling inhibits JA-dependent resistance to necrotrophs. They used salicylic acid–nonaccumulating Arabidopsis mutants (NahG) infected with *Pseudomonas syringae* pv. *tomato* DC3000 and observed a significantly increased expression of genes involved in JA biosynthesis and metabolism. Because these changes have never been observed in infected wild type plants (WT), the authors proposed that accumulation of SA during bacterial infection actively suppresses expression of JA-responsive genes. Although, major reports concentrated on the antagonistic interactions between JA and SA (Spoel et al., 2003; Tuominen et al., 2004; Li et al., 2006; Mao et al., 2007), observation of Mur et al. (2006) indicates also synergistic activity of SA and JA in potentiating the oxidative burst in tobacco. Ethylene signaling is involved in the response to mechanical damage and herbivore attacks, establishment of resistance against necrotrophic fungi like *Botrytis cinerea* and *Alternaria brassicicola* (Thomma et al., 1999; Díaz et al., 2002) and, together with JA signaling, for activation of the *plant defensin* gene (PDF1.2) upon pathogen infection (Penninckx et al., 1998; Thomma et al., 2001).

In the past years crosstalk between phytohormones has become important for the understanding of plant-microbe interactions. Navarro et al. (2008) showed that some correlations exist between gibberellin signaling and other phytohormones in the establishment of plant defence. They proved that quadruple DELLA mutants (genes known to encode negative regulators of GA signaling) react with elevated susceptibility to infection by necrotrophic fungi like *Alternaria brasicicola* or *B. cinerea* but with enhanced resistance to biotrophic pathogens like *P. syringae* pv. tomato DC3000 or *Hyaloperonospora arabidopsidis.* Changes in GA-signaling during invasion by biotrophic pathogens showed a stronger induction of the SA marker genes PR-1 and PR-2 and a drastic retardation of expression of the JA/ET marker gene PDF1.2. Although the role of cytokinins in plant-microbe interactions is mostly associated with auxin in induction of tumor formation by *Agrobacterium tumefaciens* (Sakakibara et al., 2005), in the last few years more and more information about their significance in plant immunity has become available. Argueso et al. (2012) observed that Arabidopsis plants, during infection of the biotrophic oomycete *H. arabidopsidis* isolate Noco2, were more susceptible when the exogenously applied cytokinin concentration was lower than 1 µM, but increased resistance could be detected with higher cytokinin concentrations

(>10 µM of 6-Benzylaminopurine; BAP). Additionally, they analysed response to higher level of exogenous cytokinins of *eds*16 mutants (enhanced disease susceptibility mutant) which has been mutated in *isochorismate synthase1* gene responsible for SA biosynthesis. These mutants did not show enhanced defence responses during infection of *H. arabidopsidis* and simultaneously cytokinin treatment indicating that cytokinins are dependent on SA biosynthesis and therefore may act upstream of SA biosynthesis in plant immune responses against this pathogen (Argueso et al., 2012). ABA is generally thought to be involved in negative regulation of plant defence by suppressing SAR (Yasuda et al., 2008). Nevertheless, it has been suggested that its mode of action specifically depends on individual plant-microbe interaction rather than on only pathogen lifestyle (Bari & Jones, 2009).

1.3. Auxin and its signaling mechanisms

Auxin belongs to the best known family of plant growth regulators. The term auxin comes from the greek word “auxein”, which means “to grow”. In plant developmental processes, auxin plays a role in induction of shoot apical dominance (Tanaka et al., 2006). By altering the cell wall plasticity, auxin is responsible for elongation and curvature of coleoptiles. It participates in many types of tropism (Iino & Haga, 2005), provokes cell enlargement and induces lateral root formation (Celenza Jr et al., 1995). Furthermore, it is responsible for fruit growth and influences fruit senescence (Tingwa & Yung, 1975). It has been suggested that, depending on the concentration and localization *in planta*, auxin may be responsible for molecular communication between different tissue layers by influencing metabolism of other phytohormones (Jaillais & Chory, 2010). The molecule most often identified as auxin is indole-3-acetic acid (IAA) but other indole derivatives also possess auxin-like activity (Ferro et al., 2007). Additionally, many synthetic indole derivatives are available and used in horticulture as herbicide or rooting substances, e.g. indole-3-butyric acid (IBA), indole-3-propionic acid, naphthalene acetic acid (NAA) and 2-4 dichlorophenoxy acetic acid (2-4-D) (Abad & Monteiro, 1989). IAA is synthesized in the shoot apex, leaves, cotyledons and sometimes locally in the roots but the most active production occurs in young leaves (Ljung et al., 2001). Synthesized auxin is then moved into the plant root tip via polar transport in the vascular system (Morelli & Ruberti, 2000). In shoots, auxin is

transported mostly basipetally (from the apex toward the base), whereas in the roots transport occurs basipetally in the epidermis as well as acropetally (from base toward the apex) in the central cylinder (Woodward & Bartel, 2005). The auxin polar transport is mediated mostly by efflux carriers, like PIN (pin-formed) proteins, which can quickly circulate between the plasma membrane and endosomal compartments (Geldner et al., 2001), or influx carriers, like an AUX1 (auxin resistant1 protein). Free IAA is the active form of auxin, whereas for storage or detoxification IAA is conjugated with bigger molecules like sugars, amino acids or peptides (Hangarter & Good, 1981; Woodward & Bartel, 2005). The level and ratio of free and conjugated auxin strongly depends on the plant developmental stage (Bialek & Cohen, 1989) and can be controlled reversibly by hydrolysis of IAA-conjugates or irreversibly by oxidation of IAA or IAA-conjugates (Normanly & Bartel, 1999). Conjugation of IAA to amino acids is controlled by rapid accumulation of GH3 (Gretchen Hagen3) gene transcripts encoding for IAA-amido synthetases (Staswick et al., 2005). Auxin concentrations rapidly influence expression of three families of transcripts: Aux/IAA (auxin/indole-3-acetic acid), SAURs (Small Auxin-Up RNA) and GH3-related transcripts (Woodward & Bartel, 2005). Aux/IAA proteins have been identified as short-lived and nuclear-localized negative regulators of gene expression (Kim et al., 1997). They consist of four conserved domains (Hagen & Guilfoyle, 2002). The function of domain I is still unclear, but it has been suggested that it is responsible for short-range repression (Tiwari et al., 2004), whereas domain II has been suggested to be involved in the targeting of Aux/IAA protein for ubiquitination by the SCF E3 ubiquitin ligase complex. Domain III and domain IV of the AUX/IAA protein are thought to be responsible for homodimerization with other like domains or for heterodimerization with DNA binding domains of ARFs (auxin response factors) (Kim et al., 1997). ARF can bind directly to a conserved DNA sequence in early auxin-responsive gene promoters called auxin-response element (AuxRe; Tiwari et al., 2003). ARFs contain a conserved N-terminal DNA binding domain (DBD) and often a conserved C-terminal dimerization domain (CTD). Additionally, the middle part of ARFs is known to decide about mode of the gene regulation. In transcription activators (ARF5 and ARF7), a glutamine-rich middle region can be found, whereas serine-rich (ARF2 and ARF9), serine- and proline-rich (ARF1 and ARF4), and serine- and glycine-rich (ARF3) regions are present in transcription repressors (Tiwari et al., 2003). In

absence of auxin, AUX/IAA proteins bind to ARF and repress transcription of early auxin response genes. Increase of auxin concentration provokes its targeting for ubiquitin-mediated degradation (Dharmasiri & Estelle, 2002; Woodward & Bartel, 2005). This degradation is mediated by SCF^{TIR} ubiquitin ligase. This multi-protein complex consists of three core subunits: SKP1 (S-phase kinase-associated protein 1), CUL1 (cullin) and an F-box protein (e.g. transport inhibitor response 1, TIR1). F-box protein interacts directly with Aux/IAA protein and this binding is significantly enhanced by the presence of auxin (Kepinski & Leyser, 2004). In pull-down assays using crude plant extracts and recombinant glutathionine-S-transferase fusion protein GST-IAA7, TIR1 protein has been identified as auxin receptor (Dharmasiri et al., 2005). The authors showed, that auxin enhanced interaction of TIR with Aux/IAA proteins which is required for their degradation. Proteolysis of AUX/IAA releases the repressor ARF proteins bound to the AuxRe region in early auxin response genes thereby activating their transcription (Fig. 2).

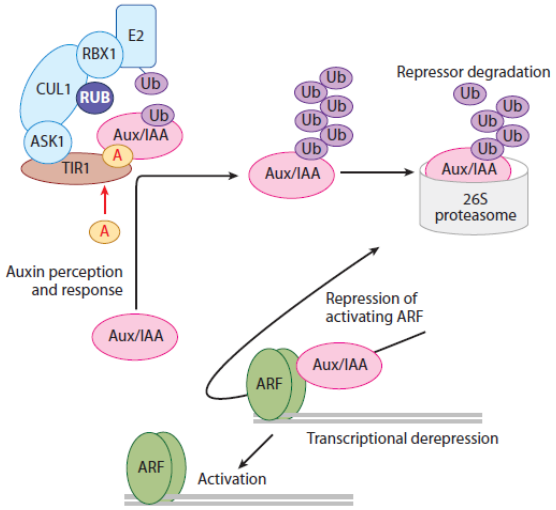

Fig. 2 Auxin SCF^{TIR} dependent signaling (from Mockaitis & Estelle, 2008). IAA activates binding of TIR to AUX/IAA, which in turn diminishes its binding to ARF DNA binding domains. The repressor, ubiquitinated by E3 ubiquitin complex, is then degraded by 26S proteasome. Degradation of AUX/IAA protein activates transcription of early auxin response genes.

1.4. Auxin crosstalk with other phytohormones and its role in plant defence

Auxin is known to be a major phytohormone in controlling plant growth. Jaillais & Chory (2010) proposed auxin to be a coordinator of the signaling crosstalk between

various plant growth regulators. Recently, this phytohormone was linked to plant defence (Bari & Jones, 2009). Externally applied auxin, mainly IAA, enhances virulence of *Agrobacterium tumefaciens* and some other biotrophic pathogens like *Pseudomonas savastanoi* (Yamada et al., 1993) or *P. syringae* pv. tomato DC3000 (Chen et., al 2007). Downregulation of the auxin signaling pathway in Arabidopsis plants results in increased susceptibility to the necrotrophic fungus *Plectosphaerella cucumerina* (Llorente et al., 2008). Additionally, the balance between auxin and cytokinins and its role in plant growth and phytopathogenicity is an oft-studied mechanism. It is known that cooperation of auxin and cytokinins is responsible for tobacco cell proliferation (Swarup et al., 2002) as well as in tumor formation during *A. tumefaciens*-plant interaction (Akiyoshi et al., 1983). They described that genes involved in the production of these phytohormones are located on the pTi plasmid of *A. tumefaciens*. High auxin concentration activates cytokinin degradation by oxidase/dehydrogenase *AtCKX6* which inhibits development of leaves. Interestingly, Lau & Yang (1973) have observed that cytokinin (kinetin) strongly enhances ethylene biosynthesis by modifying uptake of exogenously applied IAA and additionally suppresses their conjugation. Abel & Theologis (1996) showed that the higher pool of free IAA and enhanced auxin signaling act upstream of ethylene biosynthesis by the activation of the ACC synthase gene expression. Ethylene can also modify auxin content in roots. Stepanova et al. (2005; 2008) reported that the gaseous phytohormone ethylene, when supplied to the Arabidopsis roots, influences tryptophan biosynthesis, enhances expression of the tryptophan aminotransferase gene responsible for IAA production and additionally supports auxin transport into the root tips in order to obtain the maximum auxin response in the elongation zone. Moreover, an antagonistic interaction between IAA and SA has been observed by Wang et al. (2007). They showed that exogenous salicylic acid application does not influence the endogenous concentration of auxin in Arabidopsis but significantly inhibits auxin signaling by transcriptional repression of IAA receptor genes. Vert et al. (2008) reported that auxin and brassinosteroids share the same signaling components. The brassinosteroids are thought to activate phosphorylation of the transcriptional repressor auxin response factor ARF2 by brassinosteroids-insensitive gene2 (BIN2). Phosphorylation interrupts the binding of ARF2 to DNA which abolishes the ARF2 repression activity. Vert et al.

(2008) suggested that concomitant application of these both phytohormones would elevate and potentially prolong expression of target genes.

1.5. Microbial production of plant hormones

Plant associated microorganisms can produce phytohormones or phytohormone-mimics which might interfere with plant hormone homeostasis and plant defence. The best known example of phytohormone mimicry is the production of coronatine by *P. syringae* pv. *tomato* (Bender et al., 1987). Coronatine is a bacterial toxin which has a high structural similarity to the jasmonate biosynthetic precursor, 12-oxophytodienoate, and therefore is able to manipulate JA signaling in higher plants which enhances bacterial infection.

Auxin belongs to a group of phytohormones which is often produced by microbes. IAA biosynthesis was reported first in tumor-inducing bacteria like *A. tumefaciens*, *Agrobacterium rhizogenes* or *P. savastanoi* (Moris, 1986; Klee et al., 1987; Camilleri & Jouanin 1991). Its production was confirmed also in growth promoting bacteria like *Alcaligenes piechaudii, Comamonas acidovorans,* (Barazani & Friedmann, 1999) and deleterious entero- or rhizobacteria like *Klebsiella pneumonia* (El-Khawas & K. Adachi, 1999) and *Micrococcus luteus, Streptoverticillium* sp., *Pseudomonas putida*, and *Gluconobacter* sp. (Barazani & Friedmann, 1999). Additionally, the ability to produce IAA has been described in mycorrhizal fungi like *Laccaria laccata* S-238A, *Suillus bovinus* 77c (Ek et al., 1983), *Pisolithus tinctorius* (Ek et al., 1983; Frankenberger Jr & Poth, 1987) and *Hebeloma hiemale* (Gay et al., 1989). Auxin biosynthesis has been reported in biotrophic pathogens like *Balansia epichloë* (Porter et al., 1985), *Ustilago maydis* (Basse et al., 1996) and *Colletotrichum gloeosporioides* (Robinson et al., 1998), in saprotrophic *Fusarium* species (Tsavkelova et al., 2012) and some necrotrophs like *Pythium ultimum* (Rey et al., 2001) and *B. cinerea* (Sharon et al., 2007). Besides auxin, other phytohormones produced by microbes have been reported. Cytokinin production was reported for *A. tumefaciens*, smut fungus *U. maydis* (Bruce et al., 2011) and in the mycorrhizal fungi *Thelephora terrestris* and *Laccaria bicolor* (Kraigher et al., 1991). Additionally, Ho (1987) has described the ability of some isolates of *P. tinctorius* to produce auxin, cytokinin and gibberellins. *Gibberella fujikuroi, Bacillus pumilus*,

Bacillus licheniformis and *Fusarium oxysporum* are producers of high amounts of physiologically active gibberellins (Gutiérrez-Mañero et al., 2001; Hasan, 2002). In other phytopathogenic microbes like *Cercospora rosicola, Cercospora cruenta, C. pini-densiflorare* and *B. cinerea* biosynthesis of ABA was detected (Nambara & Marion-Poll, 2005; Sharon et al., 2007). Many different plant associated microbes with completely different lifestyles possess the ability to produce plant growth regulators not only *in vitro* but also *in planta.* Thus, more research is ongoing concentrating on biochemical and molecular characterization of microbial phytohormone biosynthesis pathways in order to understand their role during interactions with host plants.

1.6. Auxin biosynthesis pathways

Tryptophan belongs to the 20 standard amino acids produced by eukaryotes. Its chemical structure contains an indole functional group built by a six-membered benzene ring fused to a five-membered nitrogen-containing pyrrole ring. As tryptophan is known to be a precursor of auxin (Sarwar et al., 1992) the indole group is a building block of IAA. Nevertheless, auxin can be produced both by tryptophan-dependent and independent pathways. In the tryptophan-independent auxin route, IAA is synthesized from indole-3-glycerol phosphate in the chorismate pathway (Ouyang et al., 2000). This pathway, although rarely described in microorganisms, has been reported in the bacterium *Azospirillum brasilense* (Prinsen et al., 1993). Three microbial tryptophan-dependent routes for auxin production are named after the first intermediates and include: indole-3-pyruvic acid (IPA), indole-3-acetamide (IAM) and tryptamine (TAM) (Fig. 3).

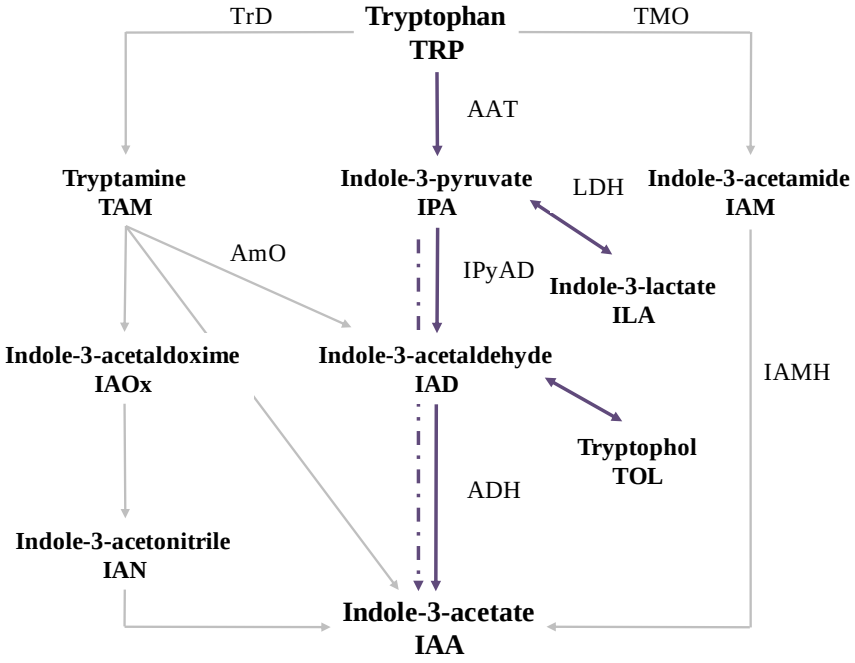

Fig. 3 Synthesis of auxin via microbial tryptophan-dependent pathways. The enzymes that catalyze the conversion are indicated near the corresponding arrow: aromatic-amino-acid transaminase (AAT), lactate dehydrogenase (LDH), indole-3-pyruvate decarboxylase (IPyAD), aldehyde dehydrogenase (ADH), tryptophan monooxygenase (TMO), indole-3-acetamide hydrolase (IAMH), tryptophan decarboxylase (TrD) and amine oxidase (AmO). The dashed line indicates a spontaneous reaction from IPA to IAA.

The most common fungal TRP-dependent pathway for IAA production is mediated by indole-3-pyruvic acid (IPA). The IPA intermediate is a product of tryptophan transamination. In the smut fungus *U. maydis*, two tryptophan aminotransferases have been described (*tam1, tam2* Reineke et al., 2008) which belong to a gene family of aromatic-amino-acid transaminases (AAT). The conversion of IPA into indole-3-acetaldehyde (IAD) is either a nonenzymatical spontaneous reaction or is mediated by indole-3-pyruvate decarboxylase (Koga et al., 1992; Costacurta et al., 1994). The final step of IAA production in this pathway is coordinated by aldehyde dehydrogenases (Rao et al., 2010). Until now, only two fungal genes encoded for aldehyde dehydrogenases (*iad1, iad2*) from *U. maydis* (Basse et al., 1996) have been molecularly characterized as being responsible for this conversion step. The IAM-mediated pathway is known to be the most common IAA production pathway in pathogenic bacteria (Manulis et al., 1998). It has been reported also in some fungi like *C. gloeosporioides* f. sp. *aeschynomene* (Robinson et al., 1998), *U. maydis* (Reineke et al., 2008) or *Fusarium* species (Tsavkelova et al., 2012). IAM is produced from tryptophan by tryptophan monoxygenase, encoded by the *iaaM* gene, and then catalyzed by indoleacetamide

hydrolase (*iaaH* gene) into IAA and ammonia. The third route uses tryptamine as intermediate (TAM-mediated IAA production pathway; Gibson et al., 1987). Presence of this pathway has been described in growth promoting as well pathogenic *Pythium* species (Le Floch et al., 2003) and *U. maydis* (Reineke et al., 2008). TAM is obtained by decarboxylation of the tryptophan. The metabolic step leading from TAM to IAD in bacteria and fungi is controlled by amine oxidases (Hartmann et al., 1983).

On the plant side, a tryptophan independent pathway has been reported to occur in plants like maize, *A. thaliana* or tobacco (Östin et al., 1999; Ouyang et al., 2000; Sitbon et al., 2000) and to be involved in regulating later stages of embryogenesis and seed germination (Normanly & Bartel, 1999). However, the TAM-pathway is the most important plant IAA production route. The conversion of tryptophan into N-hydroxy-tryptamine is catalyzed by the YUCCA enzyme (flavine monooxygenase-like enzyme). N-hydroxy-tryptamine is further converted into either indole-3-acetaldoxime (IAOx) and then to indole-3-acetonitrile (IAN) or directly to IAD (Woodward & Bartel, 2005). The IAOx pathway is speculated to be a new route of TRP-dependent IAA production in Poaceae (grasses), Brassicaceae (cabbage group and radish), and Musaceae (banana family; Thimann & Mahadevan, 1964; Zhao et al., 2002). Additionally, other tryptophan derivatives like phytoalexin camalexin or indolic glucosinolates can be produced from IAOx, which are also involved in regulation of plant defence response (Brader et al., 2001; Ferrari et al., 2003). The IPA pathway has been reported to be present in many different plants (Truelsen, 1973). Here, the first metabolic step is also controlled by aromatic-amino-acid transaminases as referred to for example in Arabidopsis (Stepanova et al., 2008) but the final step is conducted by aldehyde oxidases (Bower et al., 1978; Tsurusaki et al., 1997; Sekimoto et al., 1998). The IAM-mediated pathway has been suggested to be present commonly in plants, but it still remains unclear how IAM is produced (Lehmann et al., 2010). However in Arabidopsis the enzyme AMIDASE1 (AMI1) was shown to be responsible for conversion of IAM to IAA (Pollmann et al., 2003).

1.7. The root endophyte *Piriformospora indica*

Piriformospora indica belongs to the order Sebacinales (Agaricomycotina, Basidiomycetes). This fungus was isolated from the rhizosphere of *Prosopis juliflora* and *Zizyphus nummularia* in the Thar Desert of Rajasthan in India (Verma et al., 1998). *P. indica* is a root endophyte which colonizes many different mono- and dicotyledonous plant species and promotes host growth (Fig. 4), seed production and enhances resistance against biotic and abiotic stresses (Varma et al., 1999; Weiss et al., 2004; Waller et al., 2005; Deshmukh et al., 2006; Shahollari et al., 2007; Baltruschat et al., 2008; Sherameti et al., 2008; Achatz et al., 2010; Sun et al., 2010).

Mock treated + *P. indica*

Fig. 4 Growth promotion on barley plants triggered by *P. indica*. Picture was taken at 28 dai by Y. Ding.

The colonization strategy of *P. indica* resembles in some ways that of ectomycorrhizal fungi by creating a fungal hyphal sheet around the root, but without colonizing the root tip. Additionally, *P. indica* is able to grow inter- and intracellularly, but never produces specialized structures. Interestingly, in contrast to the mycorrhizal fungi, Peškan-Berghöfer et al. (2004) showed the ability of *P. indica* to establish a mutualistic interaction with the model plant *Arabidopsis thaliana*. Jacobs et al. (2011) have extended the knowledge about this interaction. They have shown a biotrophic relationship of *P. indica* with Arabidopsis roots during the first 3 days after inoculation (dai), which is then followed by an active cell death associated phase induced by ER stress at later time points (Qiang et al., 2012). Due to the availability of a wide range of mutants, Arabidopsis is widely used in *P. indica* studies (Stein et al., 2008; Vadassery et

al., 2008; Camehl et al., 2011; Jacobs et al., 2011). Nevertheless, research using the agriculturally important barley plant (*Hordeum vulgare*) is also often reported (Baltruschat et al., 2008; Achatz et al., 2010; Molitor et al., 2011). During *P. indica* interaction with barley, pear-shaped chlamydospores germinate on the root surface. The stylet, generated on the hyphal tip, facilitates penetration of the first epidermal cell and subsequent establishment of biotrophic interaction. During this interaction fungal hyphae are surrounded by an intact plant plasma membrane (Schäfer et al., 2009; Zuccaro et al., 2011). The biotrophic interaction lasts 3 to 5 days after chlamydospores germination (Zuccaro et al., 2011). In this phase about 10% of fungal genes encoding putative small secreted proteins (SSP) are induced (Zuccaro et al., 2011). The biotrophic phase is followed by the cell death associated phase with enhanced colonization of dead cells and production of intracellular chlamydospores. Interestingly, Henry & Deacon (1981) have described that the cells of the first cortex layers of 8 – 11 days old barley plants undergo a natural apoptosis process called root cortex cell death (RCD). At this time point, expression of *P. indica* hydrolytic enzyme is induced, suggesting a switch to saprotrophic nutrition (Y. Ding, U. Lahrmann, A. Zuccaro, personal communication). On the other hand, Deshmukh et al. (2006) described that the expression of the cell death repressor BAX inhibitor-1 (*HvBI-1*) in *P. indica* colonized roots was significantly reduced during the cell death associated phase (at 7 days onward). Thus, the authors suggested that downregulation of this gene is one of the key mechanisms in order to establish an efficient development of *P. indica* in barley roots. This duality in lifestyle and the fact that *P. indica* is readily cultivated on many different synthetic media make this fungus an interesting and suitable system to study endophytic interactions with plants.

1.7.1. Role of phytohormones during *P. indica*-plant interactions

Camehl et al. (2010a, b) and Khatabi et al. (2012) have shown that manipulation of ethylene biosynthesis and signalling changes defence responses to *P. indica* in both plants barley and Arabidopsis. The fungus colonized more efficiently mutants which are impaired in ethylene signaling (*ein2-1 and eil1*) at 14 dai (Camehl et al., 2010a), whereas the colonization of mutant *ein2-1* at the biotrophic phase (3 dai) was significantly lower (Khatabi et al., 2012). Additionally, use of the ethylene signaling

inhibitor - MCP (1-methylcyclopropene) suppressed penetration of Arabidopsis roots at 3 dai, and of barley roots at 7 dai. Ethylene response factors (ARF-9 and -14) proved to be stimulated by *P. indica* during the interaction with Arabidopsis roots, suggesting that suppression of PR-1 and PR-2 expression is required for the establishment of the mutualistic association (Camehl et al., 2010b). Microarray data of colonized barley roots (Schäfer et al., 2009; Khatabi et al., 2012) showed upregulation of genes involved in ABA (1 dai), ethylene (3 dai), and brassinosteroids production (3 dai) and signaling (3 and 7 dai) as well as in auxin production (3 and 7 dai). Moreover, Jacobs et al. (2011) have observed crosstalk communication between the GA and SA signaling pathways. *P. indica* colonized the roots of the quintuple-DELLA mutant more efficiently during the cell death associated phase (7 dai) than during biotrophy (3 dai). Expression analyses of the SA marker CBP60g (Calmodulin (CaM) Binding Protein) in this mutant revealed its upregulation at both time points (Jacobs et al., 2011). This correlates with findings from Bari & Jones (2009) that enhanced SA signaling increase resistance to biotrophs. In addition, research by Stein et al. (2008) indicated a role of JA signaling in *P. indica*-induced powdery mildew resistance. Besides the possibility to manipulate the host phytohormone balance, *P. indica* is able to secrete cytokinins and auxin in axenic culture. However, JA and ABA have not been detected under the condition tested (Sirrenberg et al., 2007; Vadassery et al., 2008).

1.8. Aim of this study

Beside a cardinal role in coordination of many developmental processes in the plant, auxin signaling has recently been shown to be involved in the induction of plant susceptibility to biotrophic and hemibiotrophic microbes (Robert-Seilaniantz et al., 2007). The beneficial root endophyte *P. indica* induces growth in many different hosts and is able to produce auxins (Sirrenberg et al., 2007; Vadassery et al., 2008). In order to clarify the role played by fungal-derived auxins in the mutualistic interaction with barley, I concentrated on the analysis of the *P. indica* biochemical pathways involved in IAA production. Barley, which is one of the top seven grains in the world (Food and Agriculture Organization of the United Nations; FAO), was chosen as a model plant. The benefits of using barley for this study are the ease with which it can be cultivated in both sterile and greenhouse conditions, as well as the ability to establish a long-lasting

beneficial interaction with *P. indica*. Thus, the main goals of this work were to identify *P. indica* genes involved in auxin production and to characterize them functionally in order to verify if fungal-derived auxin is involved in plant growth stimulation and/or in the establishment and maintenance of the biotrophic interaction with the host. Additionally, in order to perform functional analyses I established and optimized some basic molecular tools for this fungus, such as PEG-mediated transformation and a functional GFP reporter system.

2. Results

2.1. Establishment of a GFP reporter system for *P. indica*

For functional analyses of the mutualistic interaction between *P. indica* and its hosts, an efficient GFP reporter system was needed. Successful expression of heterologous genes by a host cell requires proper recognition of the promoter sequence by the transcriptional machinery. Because in our hands, the use of transformation vectors (pBGgHg and pAN7-1, for details see 4.1.2.2) containing heterologous promoters (*Agaricus bisporus* GPD and *Aspergillus nidulans* GPDA, respectively), never produced stable *P. indica* transformants, the *P. indica* promoter of the glyceraldehyde-3-phosphate dehydrogenase gene (GPD), known to possess a strong and constitutive expression, was used for the construction of new vectors. The PiGPD promoter sequence was obtained from genomic DNA using an inverse PCR (iPCR) approach (Fig. 5, paragraph 4.4.3), before availability of genome sequences (Zuccaro et al., 2011). Additionally, the sequence of the PiTEF promoter, already available in our group, was used. In total five different vectors for GFP expression containing the hygromycin resistance cassette (pTGTh, pMZGFP, pTGFPh, pToGFP, pGOGFP, for description see paragraph 4.1.2.2) were produced which combined the different *P. indica* promoters (PiTEF and PiGPD) with GFP variants including a *P. indica* codon optimized version of the enhanced GFP. Transformation of these vectors in *P. indica* was achieved using a PEG-mediated transformation approach. Effect of temperature and medium composition on *P. indica* growth was tested (Fig. 6). Although CM (complete medium) is commonly used for *P. indica* cultivation (Käfer et al., 1977), fungal growth on MYP medium was significantly faster (Fig. 6). Thus, MYP medium was supplemented with 0.3 M sucrose and used as top and bottom medium for transformation. In contrary to transformants obtained on CM, which needed 14 to 16 days of growth before transfer to new plates, transformants on MYP medium could be transferred onto fresh medium 6 to 8 days after the transformation process.

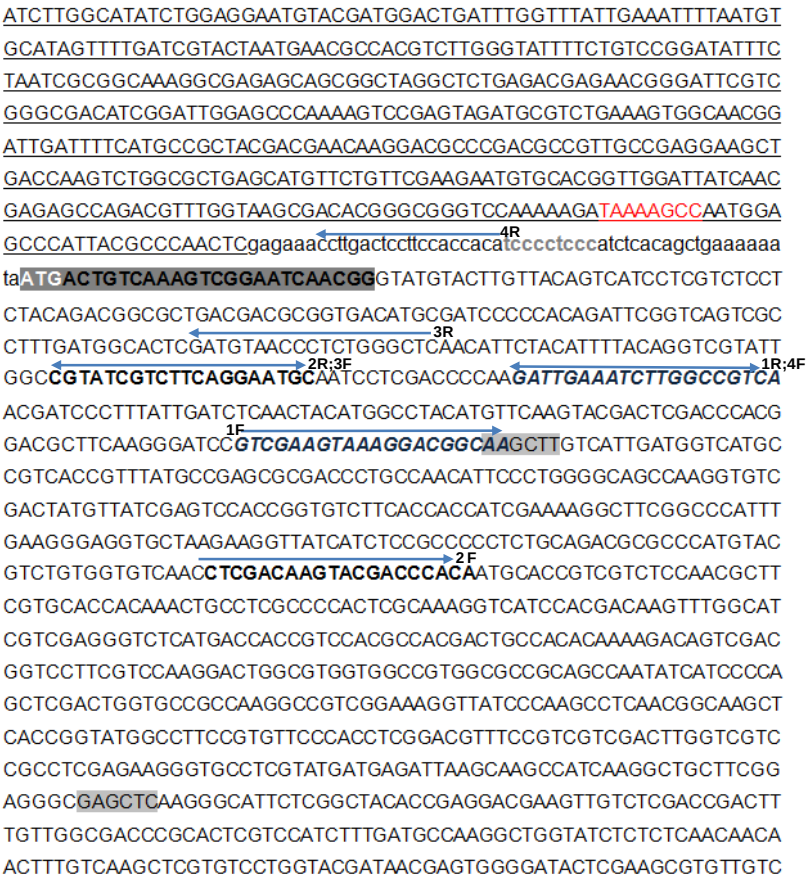

Fig. 5 Analysis of GPD promoter sequence of *P. indica*. The promoter sequence is underlined. Putative TATA box is shown in red and putative CT stretch in grey. Small letters indicates 5′ untranslated region (utr) and the start codon is shown in white font colour. The arrows indicate primer sites and the direction of synthesis. The numbers show the order of used primers. Grey boxes show restriction sites (*Hind*III and *Sac*I).

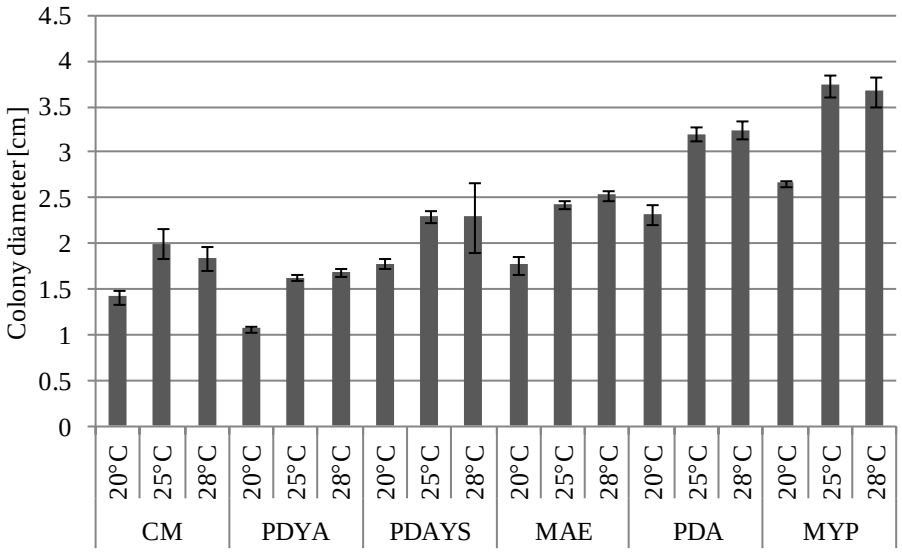

Fig. 6 Effect of medium composition and temperature on *P. indica* growth was tested on solid medium. Colony diameter was measured after 4 days. Cultures were inoculated with stamps of 0.5 cm in diameter. Standard error was calculated from three technical repetitions. The test was repeated twice with similar results.

Genome integration of the vectors was verified using Southern blot analyses with a DIG-labelled probe targeting the introduced hygromycin resistance gene (Fig. 7A). Additionally, production of the GFP protein was confirmed by Western blot analysis with a GFP specific antibody (Fig. 7B). Southern blot analyses indicated that multiple copies of the vectors integrated into the genome of *P. indica*, and Western blot analysis confirmed the production of GFP for *P. indica* strains carrying the pTGTh, pTGFPh, pToGFP, pGoGFP and pMZGFP vectors.

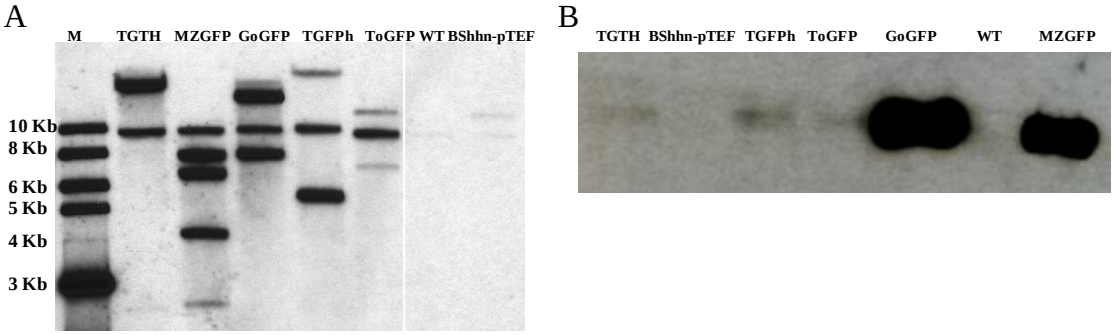

Fig. 7 Southern blot and Western blot analyses of representatives GFP strains. A: Southern blot analysis. Genomic DNA from ten-day-old liquid cultures was digested overnight with *Sac*I and separated on 0.8% TAE agarose gel for 3 h at 80V. DNA from transformant carrying vector pBshhn-TEF was used as positive control. DNA from wild type strain served as negative control. M – 2-log DNA marker. DIG-labelled hygromycin fragment was used as a probe. Minimal size calculated for the correct insertion: TGTh – 8098 bp; MZGFP – 7531 bp; GoGFP – 2667 bp; TGFPh – 6494 bp; ToGFP – 3959 bp. B: Western blot analysis with extract of total protein of representatives GFP strains. Wild type strain (WT) and transformant carrying vector pBshhn-TEF were use as control.

Confocal microscopy analyses of independent transformants for each construct indicated that *P. indica* strains carrying the pGoGFP vector, with the *P. indica* codon optimized GFP sequence had the strongest fluorescence. All constructed vectors were additionally transformed in *U. maydis* SG200 strain to confirm their functionality (Fig. 8).

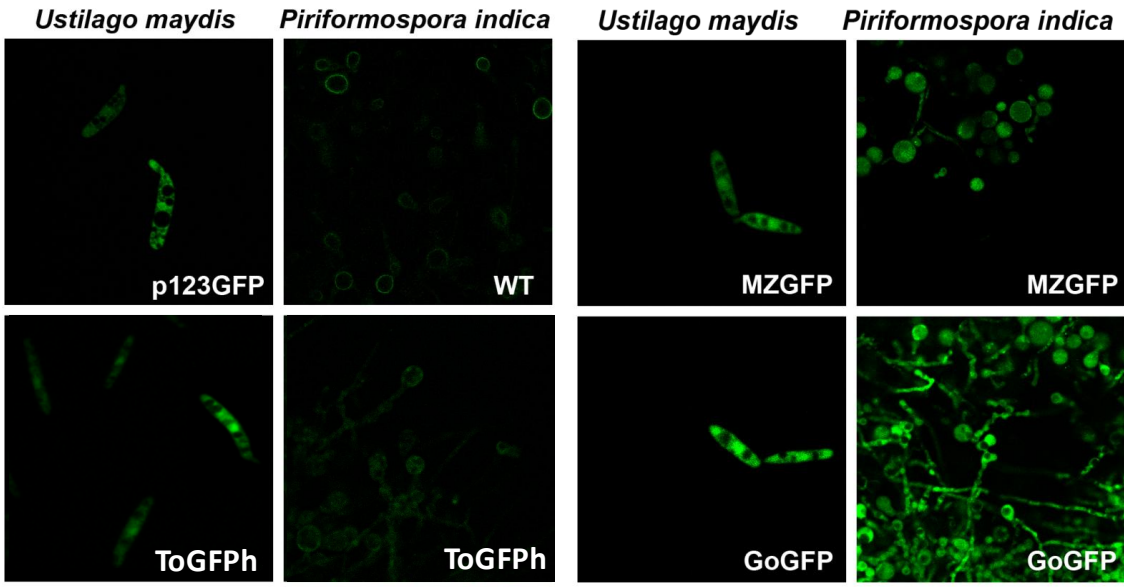

Fig. 8 Confocal microscopy analyses of *P. indica* and *U. maydis* transformants carrying different GFP constructs. Transformants carrying p123 vector were used as positive GFP control for *U. maydis.* Wild type strain of *P. indica* was used as negative control to show fungal autofluorescence.

2.2. Biological and biochemical evidence for auxin production by *P. indica*

2.2.1. Auxin activity in the culture supernatant of *P. indica*

A fast and easy test to verify the presence of auxin activity in a solution is the coleoptile elongation assay (Sirois, 1966). This test is based on the osmotic effect that auxin promotes by lowering the pH (Marré et al., 1973). This results in cell wall loosening visible as cell elongation. To verify auxin production and secretion into the medium by *P. indica*, 0.5 cm-long barley coleoptile pieces were used. These coleoptile pieces were incubated over night in *P. indica* supernatant collected from feeding test experiments performed using CM supplemented with TRP, IAD, IAA, IAM or TAM. As a positive control, 3 µM of IAA was mixed with the mycelium-free CM supernatant culture and applied to coleoptiles, which resulted in a significant elongation compared to the negative CM or TRP controls (Fig. 9). Similarly, coleoptiles incubated in supernatant from *P. indica* cultures fed with TRP or with IAA were about 20% longer than CM control (Fig. 9).

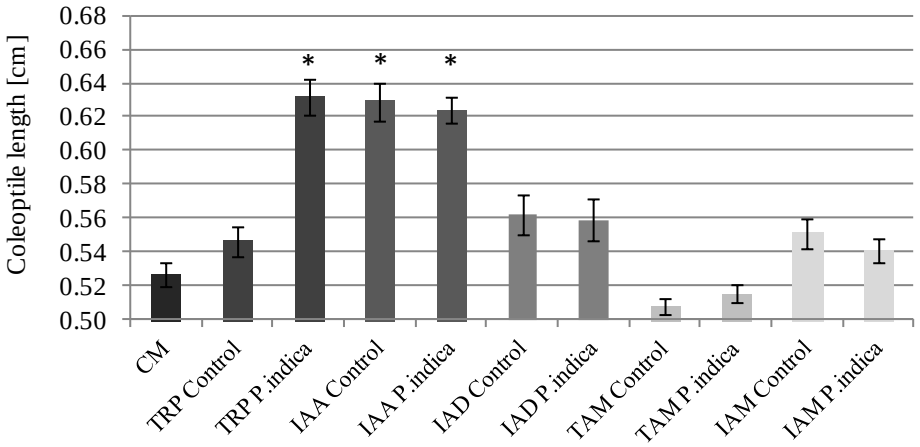

Fig. 9 Auxin activity in *P. indica* supernatant confirmed by barley coleoptile elongation test. *P. indica* cultures were propagated in CM supplemented with different indole derivatives. As a control, indole derivatives were added into mycelium-free supernatant. Significant elongation was observed in the IAA control as well as in *P. indica* culture supernatant after TRP or IAA treatments. Abbreviations: IAD - indole-3-acetaldehyde; TAM - tryptamine; IAM - indole-3-acetamide. Error bars represent standard errors of the mean (n=50). Asterisks indicate significant differences from control treatment with tryptophan (TRP Control) ($P<0.01$).

Supernatants from cultures fed with TAM, IAM or IAD (each at 0.5 mM final concentration) acted on coleoptile elongation only in a range similar to that of the control tryptophan treatment, suggesting that these derivatives could not be directly used by *P. indica* for auxin production (Fig. 9).

Auxin activity can also be measured by the auxin dose–response assay based on the ability of cotyledon explants to initiate root formation under auxin pressure (Wang et al., 2005). Extracts from culture supernatants of *P. indica* and *Sebacina vermifera* (MAFF 305830) after tryptophan feeding experiments (for details see paragraph 4.3.10) confirmed the production of auxin-like metabolites by these fungi. A production of 4.9 and 6.5 roots on average per explant was achieved after 12 days of incubation (Fig. 10). In the control cotyledon explants cultivated on ½ MS medium only callus formation was observed.

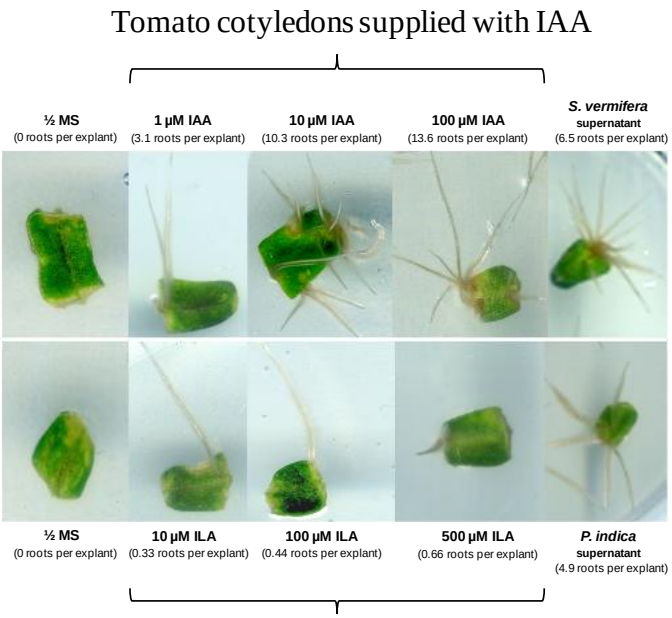

Fig. 10 Auxin-like activity shown as the ability to induce root formation on tomato cotyledons. Halves of excised tomato cotyledons were placed on ½ MS medium supplemented either with 1 µM, 10 µM and 100 µM IAA or with 10 µM, 100 µM and 500 µM ILA and cultivated for 12 days. Control treatments on ½ MS medium induced only callus formation. Addition of extract from culture supernatant from *P. indica* (P.i.) and *S. vermifera* (S.v.) after tryptophan feeding resulted in induction of roots formation.

Additionally, weak auxin activity was observed after treating the tomato cotyledon explants with ILA, a side product of IAA biosynthesis (Fig. 10). At concentrations higher than 500 µM ILA was toxic for the cotyledons and explants turned chlorotic after a few days of incubation.

2.2.2. Effect of exogenously applied auxin on *P. indica*

Exogenously applied auxin promotes growth and root formation of many different plants and triggers microbial growth (Vinklárková & Sladký, 1978; Tsavkelova et al., 2007). Although these effects are concentration-, species- and sometimes even strain-dependent, most changes are observed at an IAA concentration range of 1-10 µM. Influence of exogenously applied auxin on *P. indica* growth was tested on solid medium as well as in liquid culture. No significant changes in growth (colony diameter or dry biomass) were observed after fungal cultivation with 1 µM and 10 µM IAA. However, 100 µM IAA drastically reduced growth (Fig. 11).

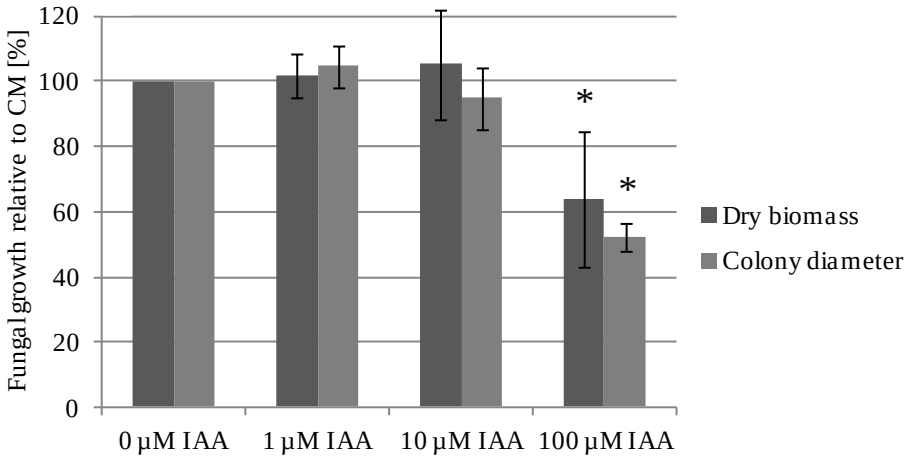

Fig. 11 Effect of exogenously applied IAA on *P. indica* growth. *P. indica* was cultivated in liquid and on solid CM. Data represent dry biomass (liquid culture, dark gray bars) and colony diameter (solid medium, light gray bars) of IAA-treated cultures relative to the untreated CM control. Biomass was collected 7 dai, simultaneously the diameter of colonies plated on 3 plates supplemented with 0 µM, 1 µM, 10 µM or 100 µM IAA was measured after 14 days of growth at 28°C. Error bars represent standard errors of the mean from three independent biological repetitions. Asterisks indicate significant differences from control treatment (P<0.05).

To study the influence of IAA on the ability of *P. indica* to colonize barley roots different concentration of IAA were added to the plant cultivation medium. Barley plants cultivated on 1/10 PNM supplemented with 1 µM and 10 µM IAA showed a higher colonization ratio (136.7% and 199.5% respectively, Fig. 12) in comparison to the control plants grown on 1/10 PNM without addition of IAA. 100 µM IAA negatively affected the colonization and plant fitness (Fig. 12).

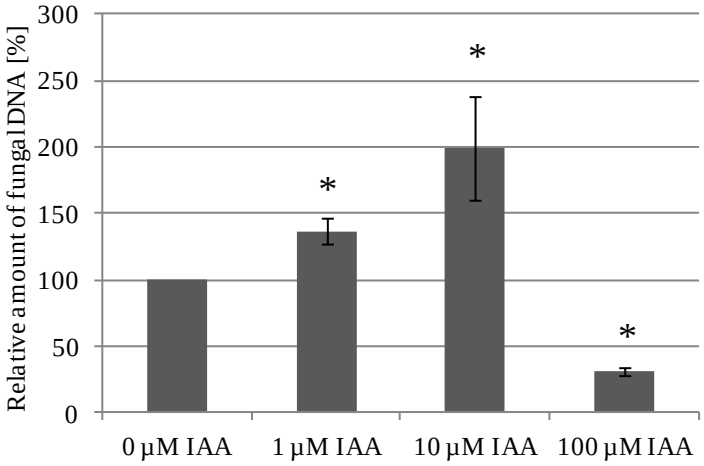

Fig. 12 Effect of exogenously applied auxin on the colonization of barley roots by *P. indica*. Three-day-old germlings were inoculated with *P. indica* chlamydospores or mock treated and cultivated for 7 days on 1/10 PNM supplemented with different concentrations of IAA. Colonization ratio in PNM control without IAA was set to 100%. Error bars represent standard errors of the mean of three independent biological replications. Asterisks represent significant differences to the control treatment (P<0.05).

2.2.3. Free IAA levels in *P. indica* colonized barley roots

Microbial interaction with plants often results in changes in IAA levels. These differences often correspond with changes in the expression of genes involved in host phytohormone signaling and production. Schäfer et al. (2009) described that during interaction of *P. indica* with barley roots, some genes involved in tryptophan biosynthesis as well as genes involved in auxin biosynthesis and signaling are upregulated at 3 and/or 7 days after inoculation, indicating that auxin production or sensing take place at the early colonization steps. To confirm biochemically an increased level of IAA *in planta*, liquid chromatography tandem mass spectrometry (LC-MS/MS) analyses of free IAA content in barley roots 3, 5 and 14 days after inoculation with *P. indica* were performed in cooperation with Dr. Lars Voll (Friedrich-Alexander University, Erlangen-Nürnberg, Germany). Colonized and mock treated barley roots were cut in two parts according to the colonization pattern of barley by *P. indica* as described by Desmukh et al. (2006). Thereby, the first 3 cm below the seed (differentiation zone) which is normally heavily colonized by *P. indica* and the rarely colonized part of the root (meristematic and elongation zone) were collected separately. Differences in the level of free IAA between colonized and mock treated barley roots could be observed at 3 dai in the differentiation zone as well as in the elongation zone (Fig. 13A, B), indicating local and systemic increased of IAA levels. No significant differences could be observed at later interaction stages.

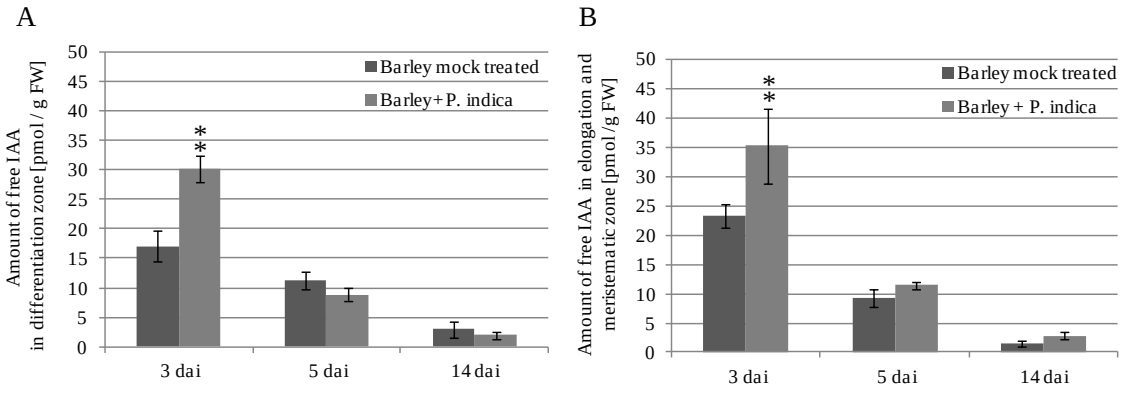

Fig. 13 LC-MS/MS analyses of free IAA content in *P. indica* colonized and non colonized barley roots. Three-day-old germlings were inoculated with *P. indica* chlamydospores (light gray bars) or mock treated (dark gray bars) and cultivated for 3, 5 and 14 days in a growth chamber. Root samples were divided into two parts: A: the heavily *P. indica*-colonized part (differentiation zone) and B: the *P. indica*-free part (elongation and meristematic zone). Error bars represent standard errors of the mean from four independent repetitions. Asterisks indicate significant differences in free IAA amount between colonized and non colonized roots ($P<0.01$).

Presence of free ILA in roots depends on the plant species (Gibson et al., 1987) and may also change upon microbial infection. Free ILA content in *P. indica* colonized roots was significantly higher than in mock treated roots at each time point (Fig. 14 A, B).

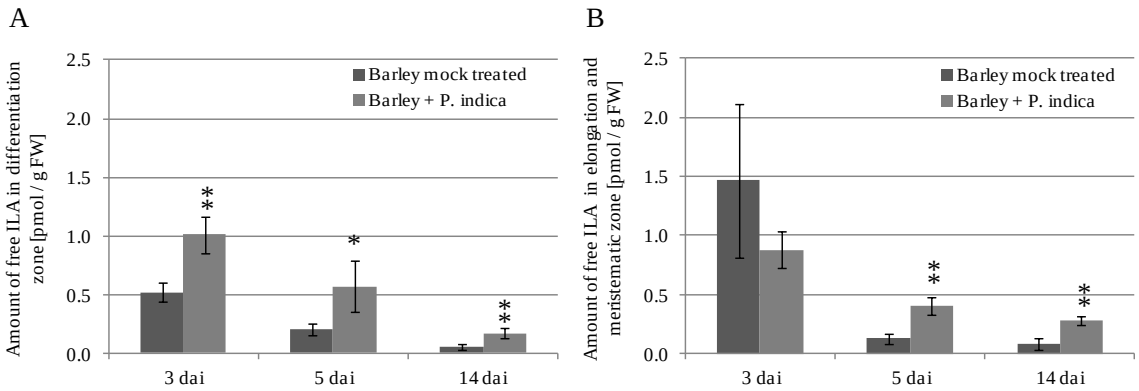

Fig. 14 LC-MS/MS analyses of free ILA content in *P. indica* colonized barley roots. Three-day-old germlings were inoculated with *P. indica* chlamydospores (light gray bars) or mock treated (dark gray bars) and cultivated for 3, 5 and 14 days in a growth chamber. Root samples were divided into two parts: A: the heavily *P. indica*-colonized part (differentiation zone) and B: the *P. indica*-free part (elongation and meristematic zone).Error bars represent standard errors of the mean from four independent repetitions. Asterisks indicate significant differences in free ILA amount between colonized and non colonized roots (** P<0.01, * P<0.05).

2.2.4. *P. indica* auxin biosynthesis pathway

To identify and characterize the auxin biosynthesis pathways in *P. indica*, feeding tests with a standard indole precursor (TRP) and other indole derivatives were performed. The supernatants were analysed using the Salkowski reagent in a colorimetric test followed by thin layer chromatography (TLC, Bric et al., 1991). The Salkowski reagent reacts with oxidized indole groups resulting in a colouring of the solution ranging from light to dark red, blue and yellow, where the intensity is proportional to the indole derivatives concentration. Representative results for the *P. indica* time course colorimetric test are shown in Fig. 15.

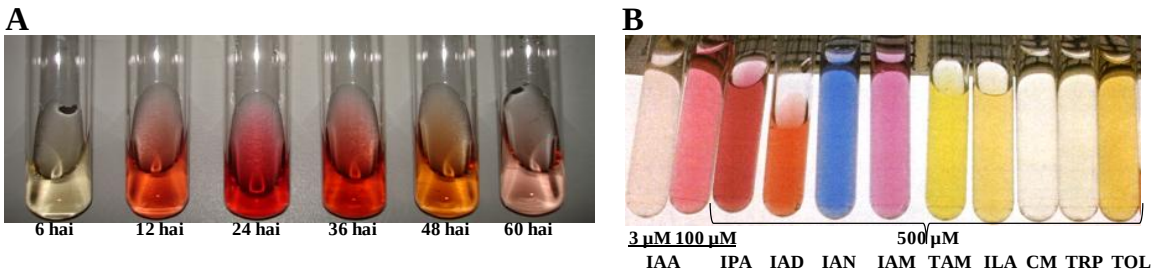

Fig. 15 Detection of auxin in *P. indica* culture supernatant using the Salkowski reagent. *P. indica* was cultivated in liquid CM supplemented with 2.5 mM TRP over a period of 60 hours.A: Culture supernatants from this time course are shown after treatment with Salkowski reagent B: Commercially available indole derivatives were added into mycelium-free liquid CM and treated with Salkowski reagent. Pictures were taken after incubation for 25 minutes in complete darkness by (A) M. Sharma and (B) M. Hilbert.

Fig. 16 shows a developed TLC plate with extracts from a time course study of *P. indica* cultures grown in liquid CM supplemented with 2.5 mM tryptophan. Using both approaches several indole derivatives were identified: tryptophan (Retention factor (Rf) 0.025), IPA (Rf 0.065), IAA (Rf 0.35), tryptophol (TOL) (Rf 0.60) and IAD (Rf 0.70). No spots for IAM (Rf 0.45) or for IAN (Rf 0.75) were identified under this condition.

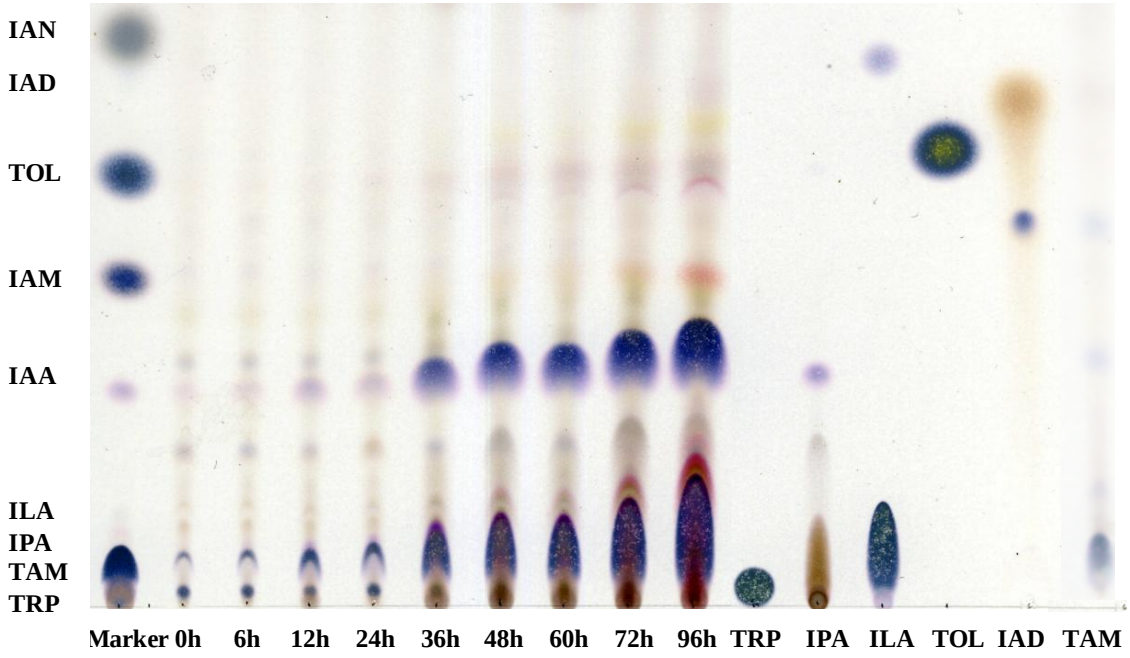

Fig. 16 TLC analysis of *P. indica* culture supernatant extracts from a tryptophan feeding experiment. Time course experiments were carried out in triplicates. *P. indica* was pregrown in CM for 1 week before induction with 2.5 mM TRP. The predominant TLC spots represent indole-3-acetic acid (IAA) (blue/violet), indole-3-lactate (ILA) (blue) and indole-3-pyruvic acid (IPA) (brown). The commercially available indoles were used as additional markers: TRP - tryptophan; IPA - indole-3-pyruvic acid, ILA - indole-3-lactic acid; TOL – tryptophol, IAD - indole-3-acetaldehyde and TAM - tryptamine. A chloroform:methanol:water mixture (84:14:1) was used as running buffer.

Because of the similar retention factor of TAM (Rf 0.045) and ILA (Rf 0.08) and similar colour of spots on the TLC plate, distinguishing between these two indole derivatives was difficult under the separation condition applied. Thus, 1 µl ILA or TAM were additionally mixed to *P. indica* 12 hai culture supernatant extract and separated on a standard TLC plate (Fig. 17A). Addition of TAM into the culture supernatant extract resulted in the appearance of an additional spot (Fig. 17A, spots number 2), indicating that this indole is not present in the supernatant. After addition of ILA, the size of the spot number 3 increased, indicating that this spot may represent ILA. This result could be confirmed using a different elution buffer (2-propanol:water, 4:1) (Fig. 17B).

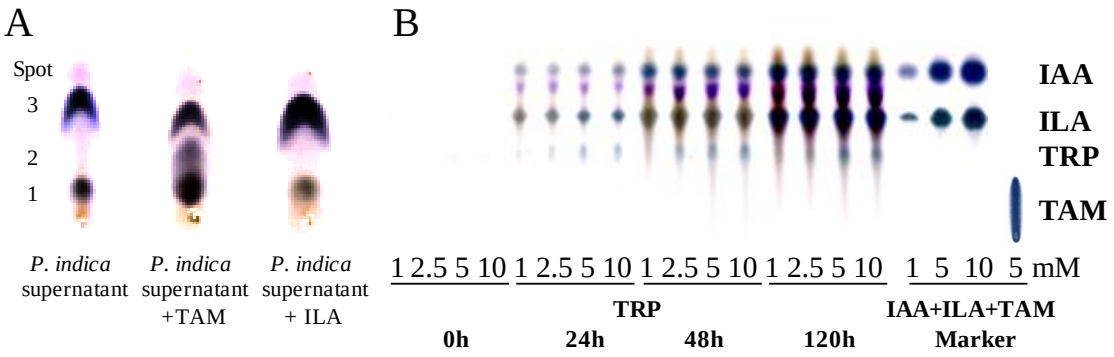

Fig. 17 Separation of ILA and TAM in *P. indica* culture supernatant extracts by TLC. A: Extract from culture supernatant after TRP treatment (12 hai) was mixed with TAM and ILA marker and separated using a chloroform:methanol:water mixture (84:14:1) as a running buffer. B: Extracts from culture supernatant from TRP induced time course studies separated in a isopropanol:water mixture (4:1) used as a running buffer.

TLC analyses have shown that IAA is produced by *P. indica* in large amount within 12 hours after addition of tryptophan (2.5 mM). Quantification of IAA and ILA production was performed by LC-MS/MS analyses in cooperation with Dr. Lars Voll. In 5 day-old cultures of *P. indica*, three times more ILA than IAA (ILA = 3.379 µmol/g FW ± 0.041, IAA = 1.196 µmol/g FW ± 0.011) was detected in the presence of 2.5 mM tryptophan. The ability to produce auxin from the precursors IAM, IPA, TAM, IAN and TOL was examined by TLC. IAA production was observed only after addition of IPA but not after addition of IAM, TAM, IAN or TOL (Fig. 18). Taken together these results indicate that *P. indica* produces IAA via the IPA pathway. No other pathways could be detected under the tested conditions.

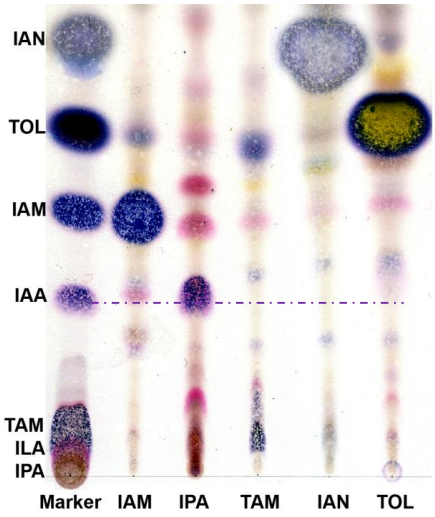

Fig. 18 TLC analysis of *P. indica* culture supernatant extracts from feeding experiments with different auxin intermediates. Supernatant extracts from *P. indica* grown for 3 days on CM supplemented with IAM, IPA, TAM, IAN or TOL separated on TLC. A spot identified as IAA (blue/violet) is visible only after addition of the intermediate IPA to *P. indica* culture. A chloroform:methanol:water mixture (84:14:1) was used as a running buffer.

2.2.4.1. Influence of cultivation conditions on the synthesis of IAA

Production of indole derivatives may change depending on the available carbon sources and/or the amount of tryptophan (Chung et al., 2003; Shilts et al., 2005). Thus, *P. indica* was cultivated in minimal medium (MM) supplemented with glucose or arabinose as a sole carbon source with four different tryptophan concentrations. Under these conditions no additional indole derivatives were detected. In glucose containing medium, *P. indica* produced more indole derivatives than in arabinose containing medium (Fig. 19). The concentration of initial tryptophan did not significantly influence IAA production during growth on glucose, while higher tryptophan concentrations (5 and 10 mM) inhibited IAA production during growth on arabinose.

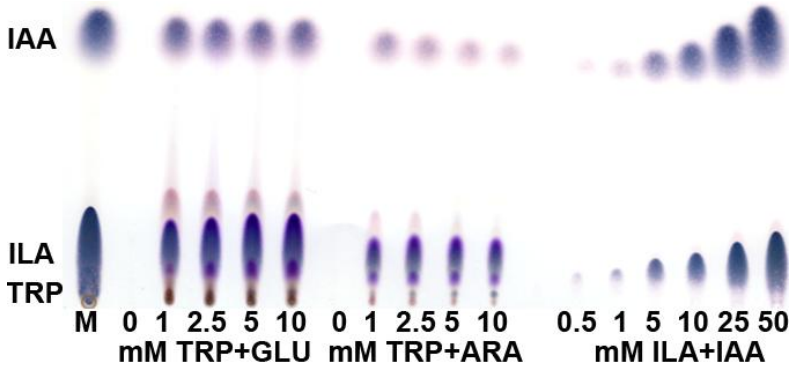

Fig. 19 TLC separation of culture supernatant extracts of *P. indica* grown for 3 days in MM supplemented with different carbon sources in the presence of different concentrations of TRP. *P. indica* was cultivated on

MM supplemented either with 2% glucose or with 2% arabinose. Extracts from culture supernatant were separated using a chloroform:methanol:water mixture (84:14:1) as running buffer. Following initial TRP concentrations were used: 0 mM, 1 mM, 2.5 mM, 5 mM and 10 mM.

2.2.5. Importance of *P. indica* endogenous bacteria in ILA production

Bacteria from the genus *Rhizobium* are known to be associated to plants and also to fungi and are often reported to be able to produce auxin (Kobayashi et al., 1995; Requena et al., 1997; Frey-Klett & Garbaye, 2005). Sharma at al. (2008) could show a tight association of the rod-shaped *Rhizobium radiobacter* PABac-DSM with *P. indica* and have reported that these bacteria can produce auxin in a tryptophan dependent manner. TLC analyses from bacterial cultures grown on different media confirmed the ability to produce IAA during growth in LB medium, but addition of tryptophan did not induced auxin production (Fig. 20). A spot corresponding to IPA was identified after tryptophan treatment but ILA was not detected. Application of ILA did not result in the appearance of additional spots (Fig. 20).

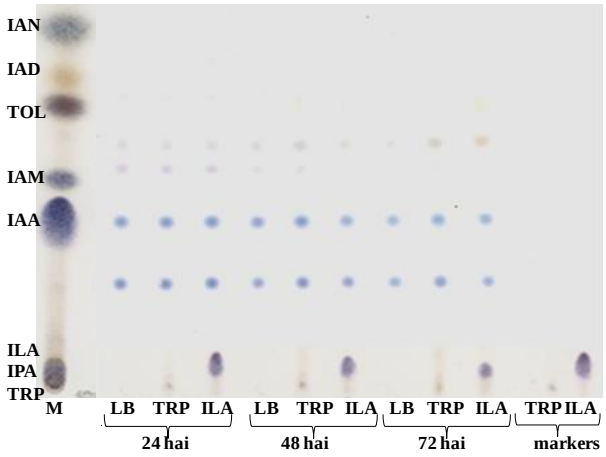

Fig. 20 TLC analysis of culture supernatant extracts from *R. radiobacter.* For the time course analysis bacteria were propagated in LB medium alone or supplemented with 0.5 mM TRP or 0.5 mM ILA**.** Extracts from cell-free supernatant were separated using a chloroform:methanol:water mixture (84:14:1) as running buffer.

2.3. Molecular analyses of *P. indica* IAA biosynthesis

Due to the release of the *P. indica* genome (Zuccaro et al., 2011), several putative candidate genes involved in auxin biosynthesis were identified.

2.3.1. Aromatic-amino-acid transaminases (AATs)

The first step in the IPA-mediated auxin biosynthesis pathway is catalyzed by aromatic-amino-acid transaminases (AATs). These enzymes are responsible for converting tryptophan into idole-3-pyruvic acid. The search for putative AATs in the genome of *P. indica* resulted in the identification of three candidate genes: PIIN_07534, PIIN_8984 and PIIN_01989. These genes display high sequence similarity to the tryptophan aminotransferase um01804 (*Tam1*) from *U. maydis* (Reineke et al., 2008) and ARO8 and ARO9 aromatic aminotransferases from *S. cerevisiae* (Iraqui et al., 1998; Table 1).

Table 1 Blastp analyses of AATs candidate genes.

P. indica **candidate gene**	**Length [amino acid]**	**Protein sequence identity [%]**		
		umTam1	**ARO8**	**ARO9**
PIIN_07534	460	43%	26%	26%
PIIN_01989	562	26%	38%	25%
PIIN_08984	570	28%	31%	28%

The *P. indica* candidate genes were tested for their responsiveness to tryptophan. Whereas expression of PIIN_08984 and PIIN_01989 was not induced by the presence of tryptophan, expression of PIIN_07534 was about 30 fold higher in tryptophan containing medium compared to CM control (Fig. 21).

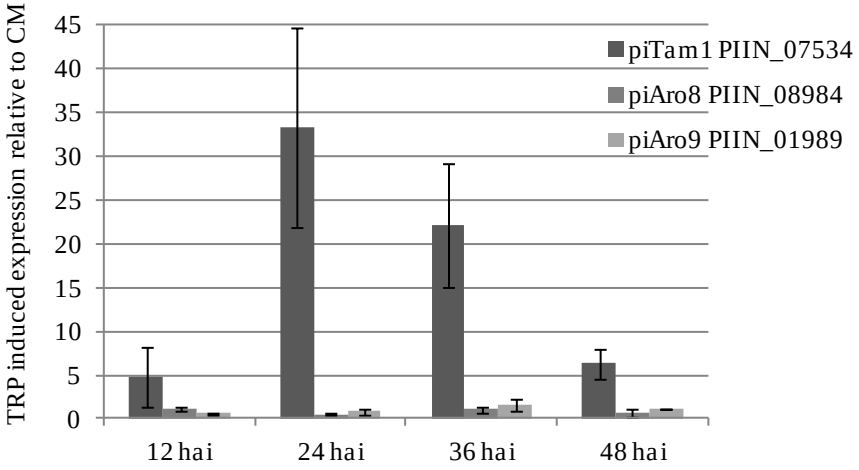

Fig. 21 Gene expression patterns of *P. indica* genes encoding AATs after tryptophan treatment. qPCR data are shown as fold changes of transcript accumulation after TRP induction calculated relative to CM control. The piTEF expression was used as internal control to normalize the data. Error bars represent standard errors of the mean from three independent biological repetitions. Abbreviation: hai – hours after induction.

Based on these results, gene PIIN_07534 was chosen for *in planta* analyses. Fig. 22 shows expression of the *piTam*1 gene during the *P. indica*-barley interaction. The *piTam*1 was 8 fold upregulated at 3 dai and its expression declined after 5 dai.

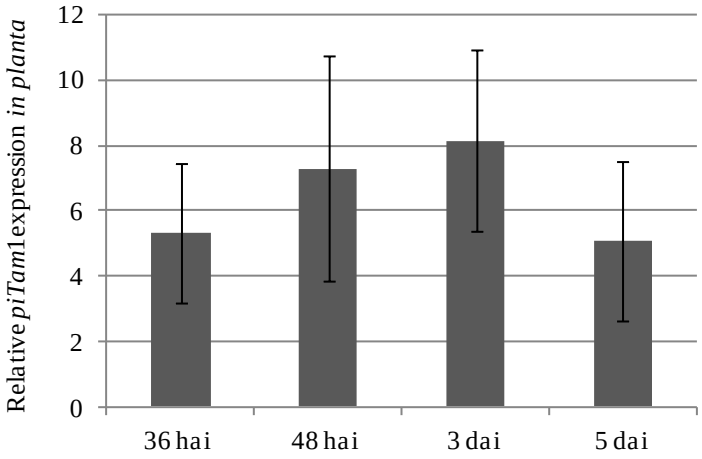

Fig. 22 Expression of *piTam*1 gene during the biotrophic interaction with barley roots. qPCR data are shown as fold change of transcript accumulation during the early biotrophic interaction with barley calculated relative to the PNM control. Error bars represent standard errors of the mean from four independent biological repetitions.

2.3.1.1. *piTam*1 promoter analyses

Tryptophan belongs to a group of amino acids commonly present in plant root exudates (Kravchenko et al., 2004). Soil microorganisms may use it as a precursor for auxin production and/or as a signal molecule for the establishment of plant-microbe interaction (Lambrecht et al., 2000). To analyze the responsiveness of the *piTam*1 gene to exogenously applied tryptophan, a GFP reporter system was constructed. Thus, the promoter of the *piTam*1 gene was cloned in front of the *P. indica* codon optimized GFP sequence (oGFP). Ten transformants (named TP1-10) were screened for GFP fluorescence intensity, using confocal laser scanning microscopy, after induction with 2.5 mM tryptophan. The TP3 transformant, with the strongest GFP activity, was cultivated on 1/10 PNM supplemented with different concentrations of tryptophan. The GFP fluorescence intensity, calculated using the corrected total cell fluorescence (CTFC; paragraph 4.5; Gavet & Pines, 2010), was proportional to the applied concentration of tryptophan between 0.1 and 1 mM (Fig. 23).

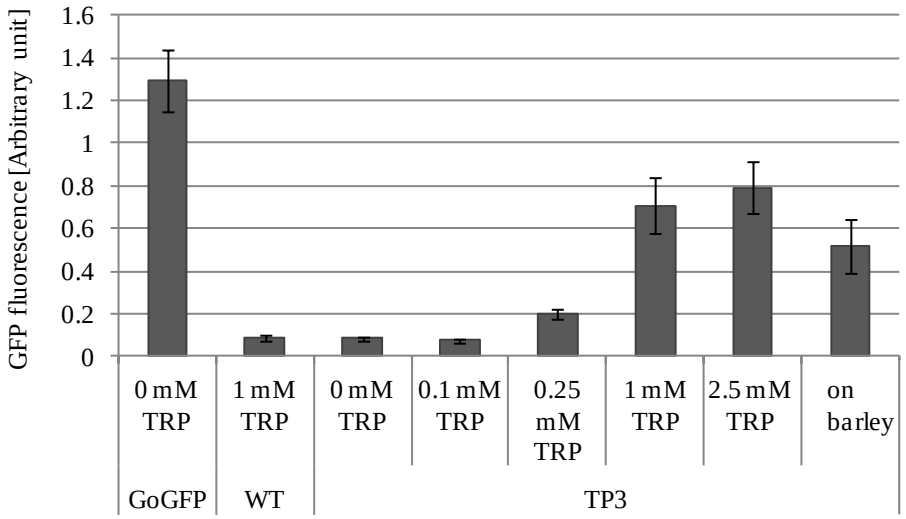

Fig. 23 Measurement of GFP fluorescence intensity using the corrected total cell fluorescence (CTCF) formula in the program imageJ. *P. indica* TP3 strain expressing GFP driven by the *piTam*1 promoter was cultivated on 1/10 PNM supplemented with different concentrations of TRP as indicated in the figure. Additionally, barley roots were inoculated with chlamydospores of TP3 strain and analyzed by confocal microscopy at 5 dai. GoGFP (with GFP under control of piGPD promoter) and wild type strains were used as controls. The same exposure time was used for all photographs.

No differences were observed between 1 and 2.5 mM tryptophan (0.71 and 0.79 units of relative fluorescence, respectively; Fig. 23), indicating that the promoter is fully activated at 1 mM TRP. Additionally, barley plants were inoculated with TP3 strain and GFP accumulation was measured 2, 3, 4, 5 and 14 dai. A strong GFP fluorescence was observed at 5 dai (Fig. 24), supporting the finding that the *piTam*1 gene is expressed during biotrophy.

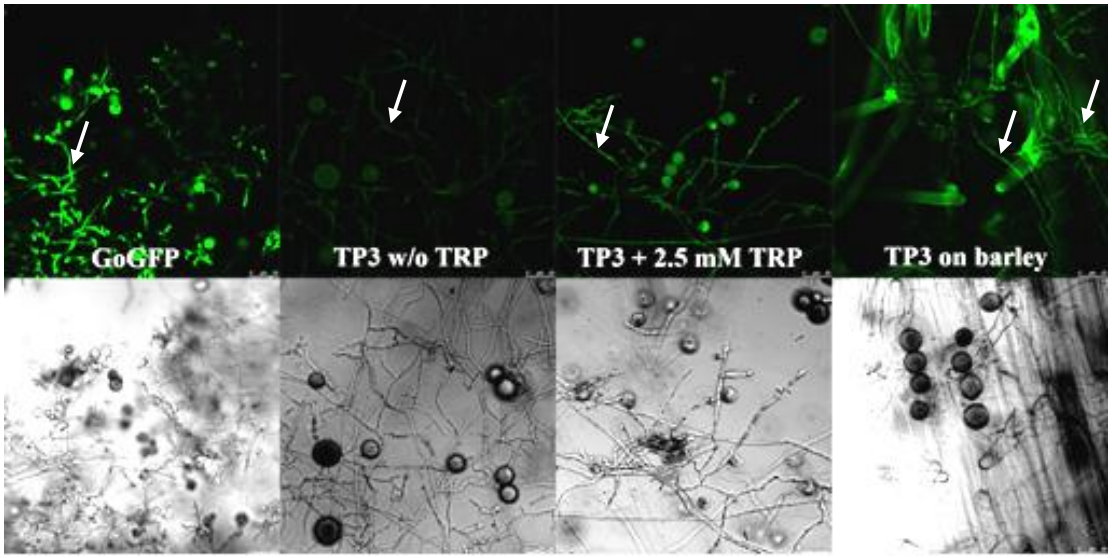

Fig. 24 Confocal microscopy analyses of TP3 strain. Transformant TP3 expressing GFP driven by the *piTam*1 promoter was cultivated either on 1/10 PNM alone supplemented with 2.5 mM tryptophan or in presence of barley

germling. The highest GFP fluorescence intensity was detected at 5 dai on planta and at 2 dai on 1/10 PNM supplemented with TRP. The WT and GoGFP strains were used as controls. The WT strain did not show any fluorescence above background under all conditions tested. From left to right: GoGFP strain on 1/10 PNM without TRP; TP3 strain on 1/10 PNM without TRP; TP3 strain on 1/10 PNM supplemented with 2.5 mM TRP; TP3 strain on barley roots. Pictures were taken using Leica SP5 confocal microscope with HCX PL APO lambda blue 20.0 x 0.70 IMM UV objective. Fluorescence was excited at 488 nm and emission was detected at 505–560 nm. Upper panel: GFP channel. Lower panel: bright field. Scale bars represent 25 µm.

2.3.1.2. Analyses of *piTam*1 RNAi strains

The importance of fungal-derived auxin on the establishment of the biotrophic interaction with barley roots was analyzed using RNAi transformants. To silence the gene PIIN_07534, a 131 bp fragment from the 3'end of this gene was cloned into pPiRNAi vector (kindly provided by Yi Ding) with the *P. indica* TEF and GPD promoters situated in a convergent arrangement (Fig. 25). The plasmid was transformed into *P. indica* wild type strain. As a control, the empty vector (pPiRNAi) was introduced into the genome of *P. indica*.

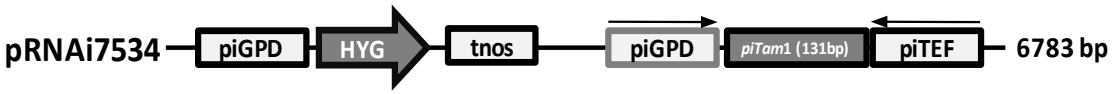

Fig. 25 Schema of pRNAi7534 vector used for knock down of *piTam*1 gene. Small black arrows indicate orientation of the *P. indica* TEF and GPD promoters.

*2.3.1.2.1. Phenotypic analyses of piTam*1 *RNAi strains*

The ability to produce auxin after tryptophan treatment was determined using TLC in five transformants, obtained from three independent PEG-mediated transformations, carrying the *piTam*1 RNAi construct (named Tam1_1, Tam2_1, Tam1_2, Tam2_2 and Tam3_1) (Fig. 26A). Three of these strains, Tam1_1, Tam2_1 and Tam2_2 impaired in auxin biosynthesis, were additionally analyzed by LC-MS/MS in cooperation with Dr. Lars Voll (Fig. 26B). About 6 fold decreased auxin production in comparison to the empty vector controls was observed. These RNAi strains displayed a less compact growth during cultivation on CM agar plates compared to empty vectors and WT (Fig. 27). This phenotype could be partially rescued by application of 10 µM IAA to the medium (Fig. 27).

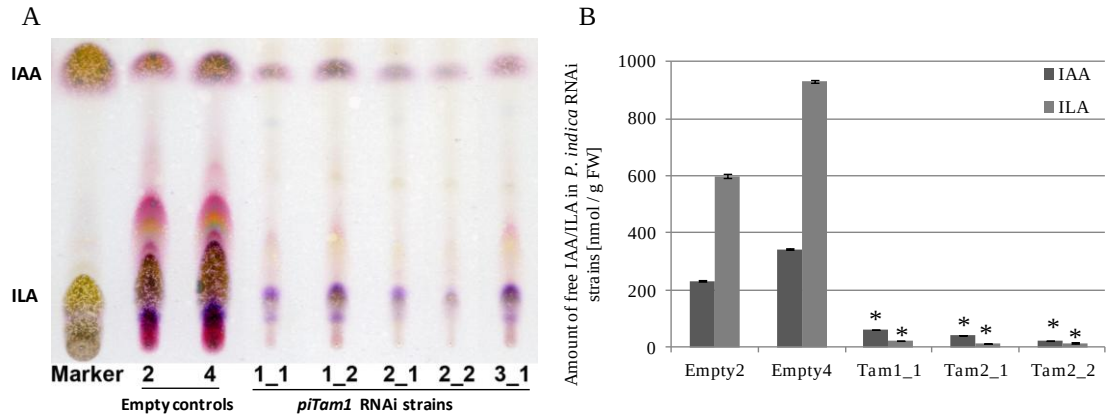

Fig. 26 Knock down of *piTam*1 gene resulted in impaired auxin production. Culture supernatant extracts from tryptophan feeding test of RNAi transformants were analyzed by A: TLC and B: LC-MS/MS. RNAi strains were grown 3 days in CM supplemented with TRP. Culture supernatant extracts from two transformants carrying empty vector were used a control. Error bars represent standard errors of the mean from three technical repetitions. Similar results were obtained in three biological repetitions. Asterisks indicate significant differences between auxin contents in extracts from empty vector control strains and from *piTam*1 RNAi strains (P<0.01).

During the cultivation of transformants on CM agar plates or liquid cultures supplemented with 2.5 mM tryptophan, the production of an unidentified pink-brownish coloured substance was observed (Fig. 27).

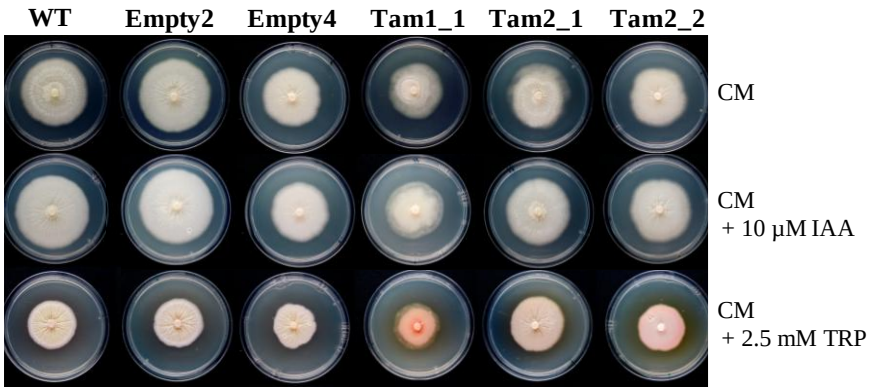

Fig. 27 Growth assay of *piTam*1 RNAi strains. Fungal mycelium plaques were placed in the middle of a CM agar plate supplemented with 10 µM IAA or 2.5 mM TRP and cultivated at 28°C in complete darkness. Pictures were taken after 14 days.

In addition, retardation of fungal growth was observed during cultivation on CM agar plates supplemented with 250 µM IAD (Fig. 28). The *piTam*1 RNAi strains grew significantly slower after application of IAD than transformants that have received the empty vector control.

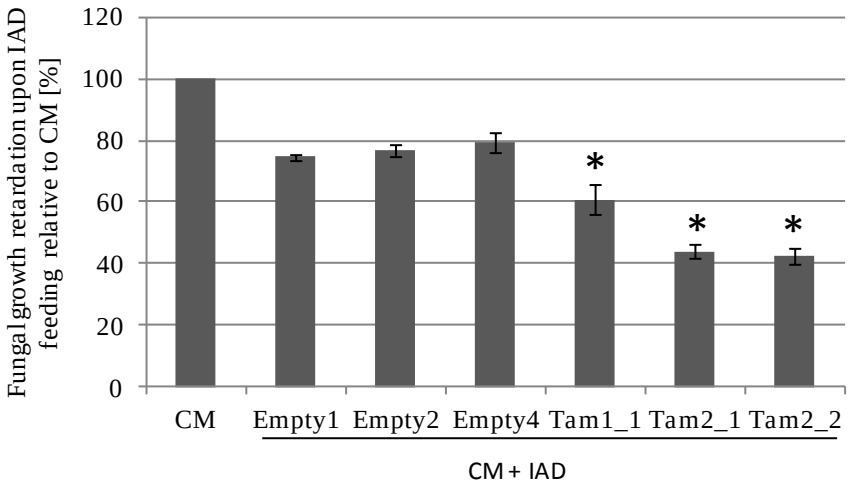

Fig. 28 Growth retardation of *piTam*1 RNAi strains cultivated on IAD supplemented medium. Growth retardation is shown as a percentage of the CM agar control and was calculated by comparing the colony diameter. RNAi strains were cultivated on CM agar plates with and without 250 µM IAD for 12 days. Error bars represent standard errors of the mean (n=3). Asterisks indicate significant growth reduction of the RNAi strains compared with the empty vector controls ($P<0.05$).

Extracted supernatants from IAD feeding test from CM liquid cultures and separated using TLC, revealed that only *piTam*1 RNAi transformants had the ability to produce auxin from this indole under this condition (Fig. 29). These results suggest a possible negative feedback of IAD on the auxin production pathway, which is abolished when the *piTam*1 gene is silenced.

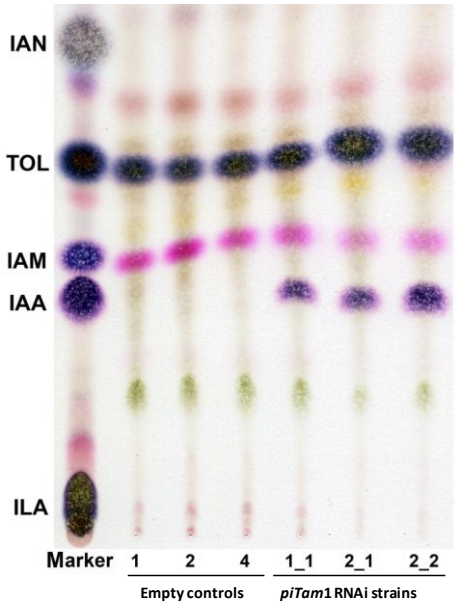

Fig. 29 Production of IAA in *piTam*1 RNAi strains after feeding with IAD. TLC analysis of extracts from *piTam*1 RNAi transformants culture supernatant incubated with 250 µM IAD for three days in complete darkness. Transformants carrying empty vector were used a control. A chloroform:methanol:water mixture (84:14:1) was used as running buffer.

2.3.1.2.2. Stability of piTam1 silenced strains

During prolonged cultivation of the RNAi strains on CM solid medium supplemented with 80 µg/ml hygromycin, some transformants became aberrant (Fig. 30).

Fig. 30 Aberrant phenotype of Tam1_1 transformant. Picture was taken from Tam1_1 transformant (8th generation) after 14 days cultivation on CM supplemented with 80 µg/ml hygromycin.

TLC analysis of supernatant extract from tryptophan feeding test of transformant Tam1_1 revealed the loss of the RNAi phenotype. Minor differences in auxin production after tryptophan induction were visible between the Tam1_1 transformant and two transformants carrying the empty vector as control (Fig. 31).

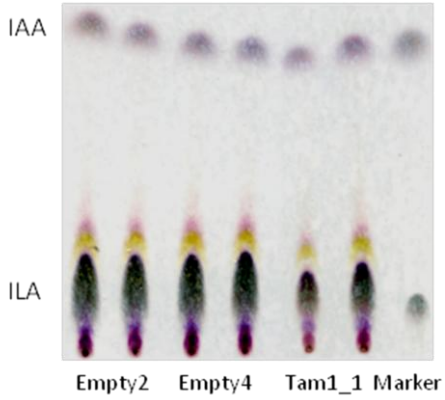

Fig. 31 TLC analysis from extracts of reverted Tam1_1 transformant after TRP feeding test. Fungal cultures inoculated with chlamydospores were propagated in CM for 7 days before TRP was supplied. Transformants carrying empty vector were used as control. Chloroform:methanol:water mixture (84:14:1) was used as a running buffer.

2.3.1.2.3. Silencing of PIIN_07534

Five transformants (Tam1_1, Tam2_1, Tam1_2, Tam2_2 and Tam3_1) were analyzed by Southern blot to verify a stable integrated insertion (Fig. 32).

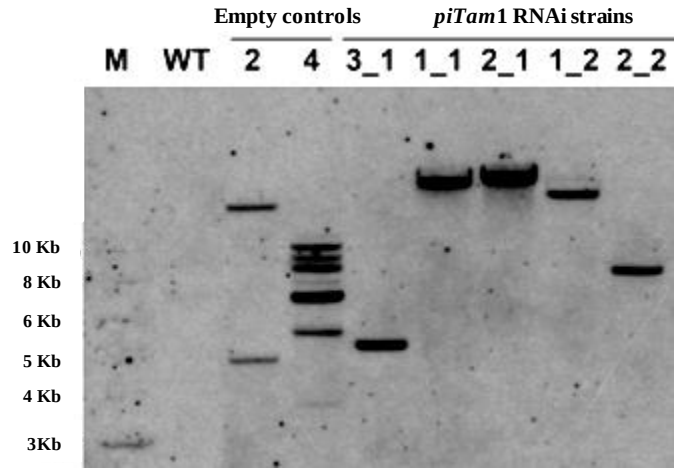

Fig. 32 Southern blot analysis of selected RNAi strains. Genomic DNA from 10-d-old liquid cultures was digested with *Nae*I (NEB) overnight and separated on a 0.8% TAE agarose gel for 3 h at 80 V. DIG-labelled hygromycin fragment was used as probe. DNA from wild type strain served as negative control.

Linearization of the vector before transformation and digestion of the DNA prior to Southern blotting with another enzyme which does not cut in the inserted vector allowed the prediction of the minimal size of insertion integrated into the genome (4251 bp). All detected fragments were bigger than 5 kb, confirming the insertion of the whole cassette. Single integration was observed by all tested transformants except for the Empty2 and Empty4 transformants. No signal was detected in the *P. indica* wild type, confirming specific binding of the DIG-labelled hygromycin probe. Three transformants displaying a different phenotype on CM than WT strain were then analyzed by qPCR. The assessment of RNAi silencing efficiency after TRP induction confirmed that *piTam*1 expression in Tam2_1 and Tam2_2 strain was silenced to a similar level (Fig. 33). Congruent with the observed revertant phenotype of the Tam1_1 strain, this transformant showed no longer silencing (Fig. 33).

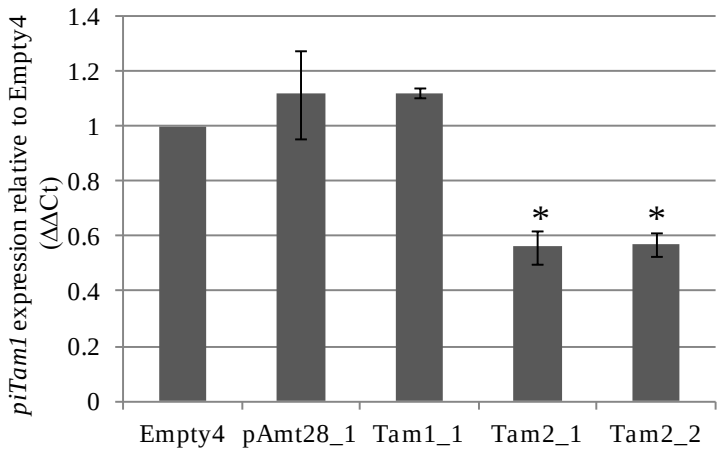

Fig. 33 Expression of *piTam*1 gene in RNAi strains. The *P. indica* RNAi strains were grown for 5 days in liquid CM prior to supplementation of 2.5 mM TRP. RNA extraction was performed 24 h after TRP treatment. Relative expression of *piTam*1 gene in RNAi transformant carrying empty vector was set to 1 (non-silencing control). Strain with downregulated ammonium transporter (Amt1_28; kindly provided by Y.Ding) was used as additional non-silencing control. Error bars represent standard errors of the mean from three independent biological repetitions. Asterisks indicate significant differences compared to the empty vector control (P<0.05).

*2.3.1.2.4. Effect of knock down of piTam*1 *gene on P. indica-barley interaction*

Because auxin was reported to induce plant susceptibility to biotrophs (Robert-Seilaniantz et al., 2007), the ability of the auxin-deficient strain Tam2_2 to colonize barley roots was tested (Fig. 34). At 3 dai the amount of measured fungal DNA from the Tam2_2 strain was significantly lower than in plants colonized by transformants carrying an empty vector. Even though during the fifth day the decreased ability to colonize barley roots was still visible, at 14 dai these differences were not significant anymore, showing the same colonization ratio for all transformants. This result indicates that auxin derived from *P. indica* is important during early interaction with barley roots.

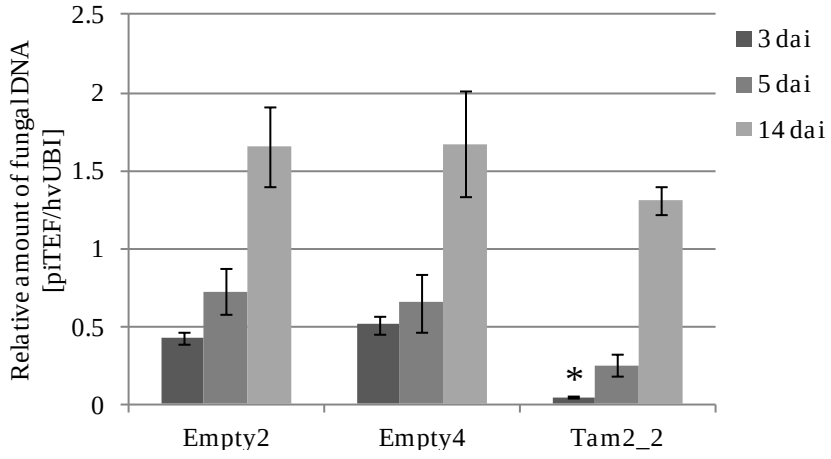

Fig. 34. Root colonization ability of RNAi strain Tam2_2. Relative amount of fungal DNA in colonized barley roots grown on 1/10 PNM at different time points (3, 5 and 14 dai) was analyzed using qPCR. Error bars represent standard errors of the mean (n=8). Asterisks represent significant differences (P<0.01).

Analysis of the barley *pathogenesis related* genes PR1b and PR10 known to be induced upon *P. indica* colonization (Schäfer et al., 2009) confirmed the impaired colonization by the RNAi strain Tam2_2. Upregulation of these genes was observed in plants colonized by the empty vector strains when compared to mock treated plants. The genes expression was significantly lower in plants colonized by Tam2_2 strain (Fig. 35).

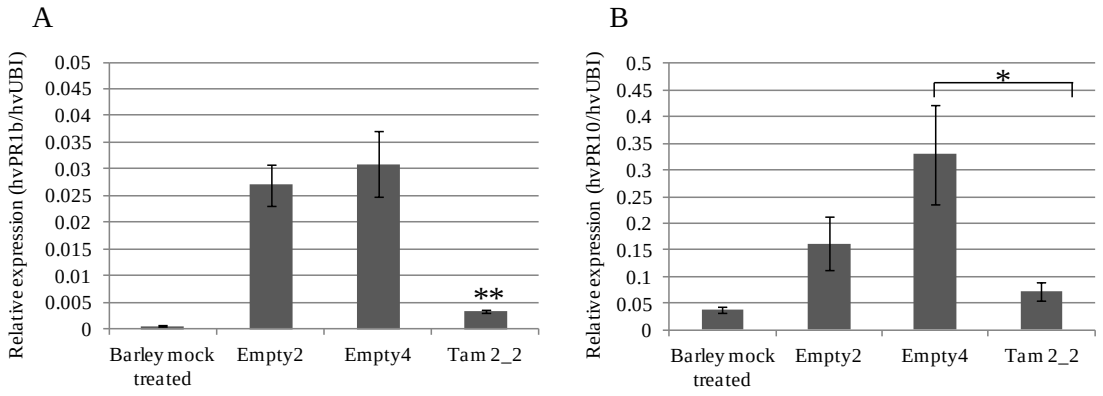

Fig. 35 Expression analyses of *P. indica*-responsive barley genes PR1b (A) and PR10 (B) in plants colonized by *P. indica* transformants during barley colonization at 3 dai. Error bars represent standard errors of the mean (n=4). Asterisks indicate significant differences in genes expression compared to plants colonized by transformants carrying empty vector (**P<0.01;*P<0.05).

To analyse the effect of fungal-derived auxin on growth promotion, barley plants were inoculated with WT, empty vector and Tam2_2 strains and leaves length and root weight were measured at 28 dai. Growth promotion effects on root weight and shoot lengths did not change significantly in comparison to the wild type and empty vector controls (Fig. 36A-C). Impairment in auxin production had no influence on the growth promotion effects triggered by *P. indica* on barley plants.

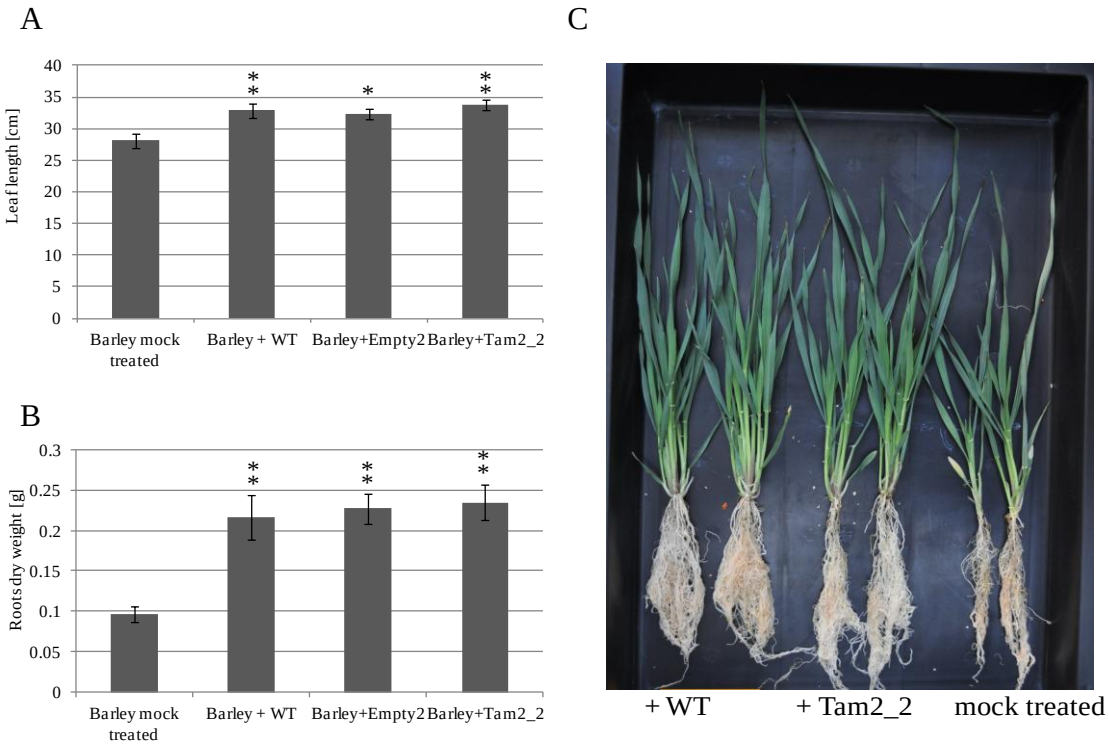

Fig. 36 Ability of RNAi transformant to trigger growth promotion effects. A: Leaf length and B: root dry weight were measured in colonized and non-colonized barley plants 28 dai. Error bars represent standard errors of the mean

(n=20). Asterisks indicate significant differences in growth of colonized plant compared to mock treated plants (**P<0.01;*P<0.05). C: Representative non-colonized and colonized barley plants by *P. indica* wild type and Tam2_2 strain. Picture was taken shortly before measurement, 28 dai by Y. Ding.

2.3.2. Aldehyde dehydrogenase

Indole-3-acetaldehyde dehydrogenase is an enzyme involved in the conversion of a formyl group into a carboxyl group and is reported to be responsible for the final oxidation reaction from IAD into IAA in *U. maydis* (Basse et al., 1996). Two candidates, PIIN_02674 (*piAdh*1) and PIIN_4899 (*piAdh*2), were identified in the genome of *P. indica*. Both candidates were predicted to possess a NAD^+-dependent aldehyde dehydrogenase domain and shared 50% and 60% identity, respectively, with the previously described indole-3-acetaldehyde dehydrogenase from *U. maydis* (Basse et al., 1996). The authors have reported that aldehyde dehydrogenase activity is carbon source dependent. The qPCR analyses showed that *piAdh*1 is glucose and tryptophan responsive whereas *piAdh*2 is mainly arabinose responsive (Fig. 37).

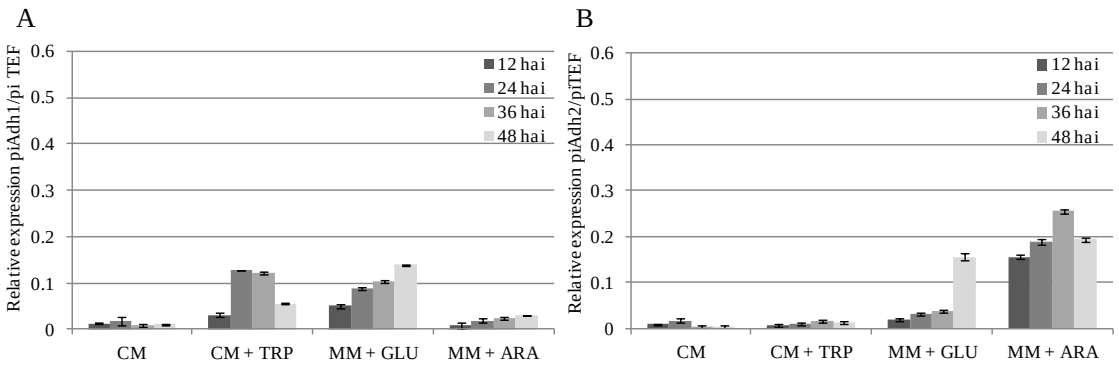

Fig. 37 Effects of tryptophan and carbon sources on gene expression level of two putative *Adh* genes. A: Expression analyses of putative aldehyde dehydrogenase gene *piAdh*1 (PIIN_02674). B: Expression analyses of the putative aldehyde dehydrogenase gene *piAdh*2 (PIIN_04899). *P. indica* cultures were propagated in CM, CM supplemented with 2.5 mM tryptophan and MM in the presence of 2% glucose or 2% arabinose at 28°C in the dark with 130 rpm shaking. Expression data were normalized using the *piTEF* gene. Error bars represent standard errors of the mean from three technical repetitions. Similar results were obtained in two independent biological repetitions.

2.3.3. D-lactate dehydrogenases

ILA is described to be a side product in the IPA-mediated auxin biosynthesis pathway in some bacteria (Fett et al., 1987; Lebuhn et al., 1997) and is one of the rarest studied indole derivatives in fungi. Only one gene, gene5, is described to be responsible for ILA production and it is located on the pTi plasmid of *A. tumefaciens* (Körber et al., 1991). Tblastn analysis using the protein sequence of gene5 revealed no similarities in the

genome of *P. indica*. The enzymatic step of ILA production by *Rhizobium meliloti* is known to be catalyzed by a NAD^+-dependent lactate dehydrogenases (Trinchant & Rigaud, 1974). A search for proteins possessing putative D-lactate dehydrogenase domains in *P. indica* resulted in 7 candidate genes: PIIN_01919, PIIN_03173, PIIN_03361, PIIN_05224, PIIN_07920, PIIN_09516 and PIIN_10496. Phylogenetic analysis classified 6 of them to be closely related to lactate dehydrogenases from other fungi, whereas only PIIN_10496 had highest sequence similarity to the bacterial lactate dehydrogenases (Fig. 38).

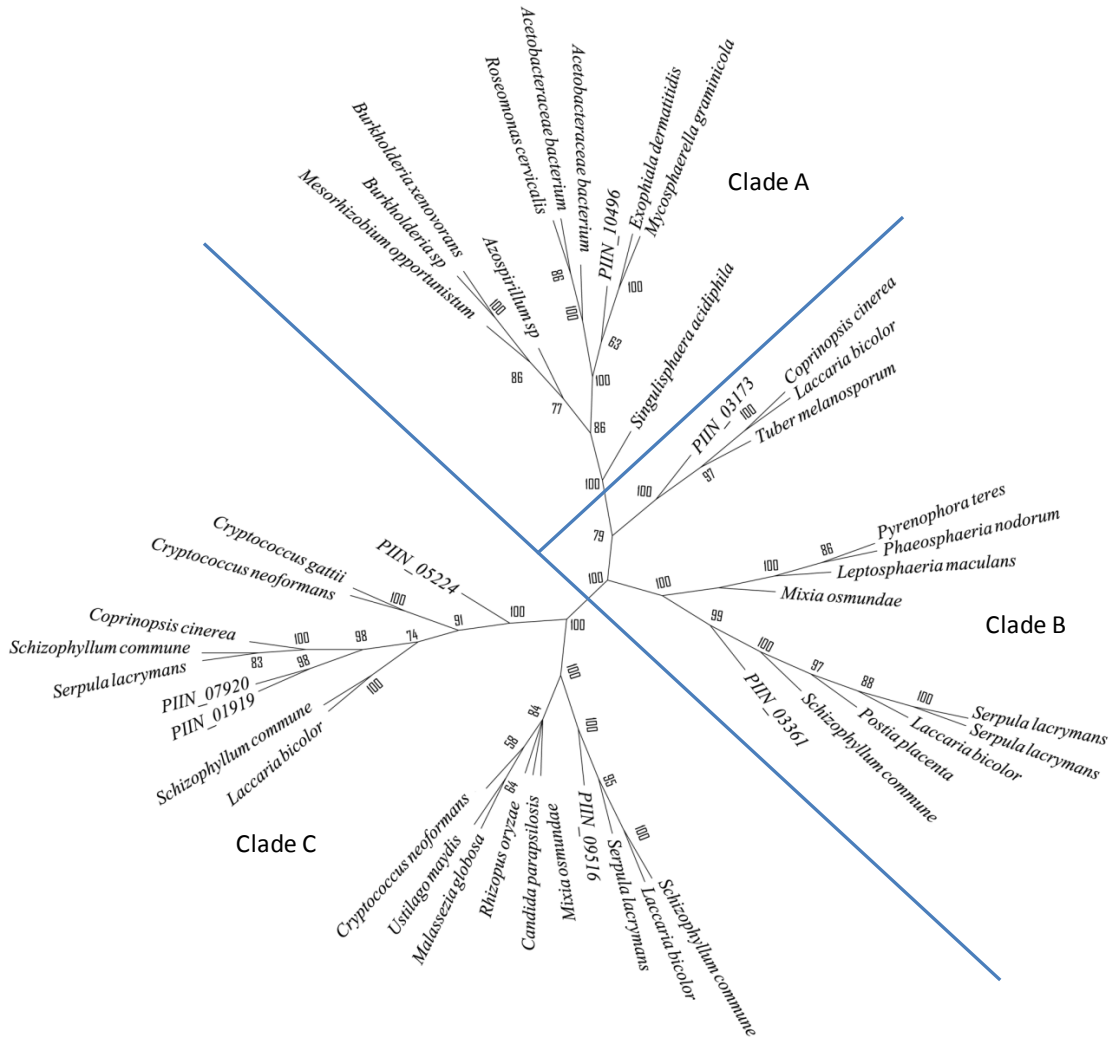

Fig. 38 Neighbor-joining phylogenetic analysis of putative lactate dehydrogenases. The *piLdh*1 (PIIN_10496) protein is positioned in clade A containing bacterial lactate dehydrogenases. Alignment of the amino acid sequence was conducted using clustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/) and neighbor-joining analysis was performed using PAUP (Swofford, 1993). Ties, if encountered, were broken randomly, distance measure = mean character difference, tree is unrooted. Bootstraps are shown close to the nodes (number of bootstrap replicates, 1000).

Three genes were predicted to be NAD^+-dependent dehydrogenases (PIIN_03173, PIIN_03361 and PIIN_10496), whereas the other four genes were predicted to contain a FAD^+-binding domain. Two NAD^+-dependent candidates, the PIIN_10496 from clade A and its closest related gene PIIN_03173, were further analyzed for tryptophan responsiveness. The PIIN_10496 (*piLdh*1) showed about 8 fold upregulation 24 hours after tryptophan induction (Fig. 39).

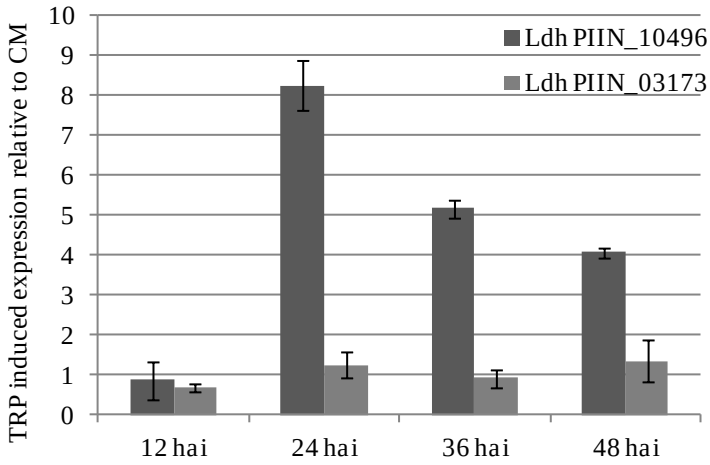

Fig. 39 *piLdh* genes expression analysis after TRP treatment. qPCR data are shown as fold changes of expression after tryptophan treatment calculated relative to control CM. The *piTEF* expression was used as internal control to normalize the data. Error bars represent technical standard errors. The time course experiment was performed two times with similar results.

2.3.3.1. Overexpression of *piLdh*1 gene in *U. maydis*

The auxin production pathway in *U. maydis* and its importance in pathogenicity and especially in tumor formation has been previously described (Basse et al., 1996; Sosa-Morales et al., 1997; Reineke et al., 2008). Nevertheless, *U. maydis* ability to produce ILA has never been reported. Feeding test with 2.5 mM and 5 mM tryptophan resulted in IAA but not in ILA production (Fig. 40).

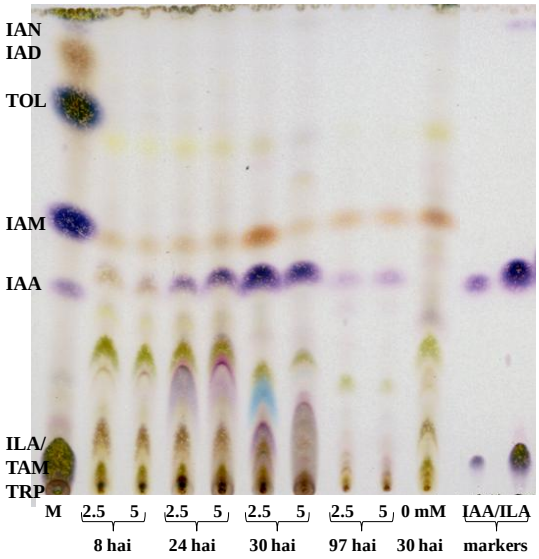

Fig. 40 TLC analysis of the time course experiment of *U. maydis* SG200 strain after tryptophan treatment. Two different tryptophan concentrations were applied into the cultures – 2.5 mM and 5 mM. Water treated culture extract was used as control. A chloroform:methanol:water mixture (84:14:1) was used as running buffer.

Thus, *U. maydis* was used for overexpression analysis of two *P. indica Ldh* candidate genes. GFP sequence from *U. maydis* overexpression vector p123 was exchanged with full-length sequence of the *piLdh*1 and *piLdh*2 genes. Transformants were verified by Southern blot analysis using a DIG-labelled carboxin probe (Fig. 41). Additionally, the expression of *piLdh*1 gene was investigated in *U. maydis* transformants using qPCR (Fig. 42).

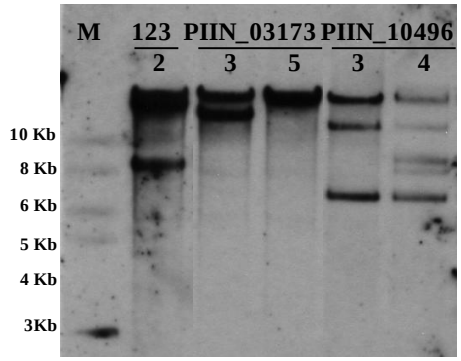

Fig. 41 Southern blot analysis of *U. maydis* transformants expressing either gene PIIN_03173 or gene PIIN_10496. Strains transformed with p123 vector were used as an additional control. Genomic DNA from overnight liquid cultures was digested with *Eco*RV (NEB) overnight and separated on a 0.8% TAE agarose gel for 3 h at 80 V. DIG-labelled carboxin fragment served as a probe. M - DNA marker 2-log ladder. Calculated minimal size of correct insertion is 4341 bp.

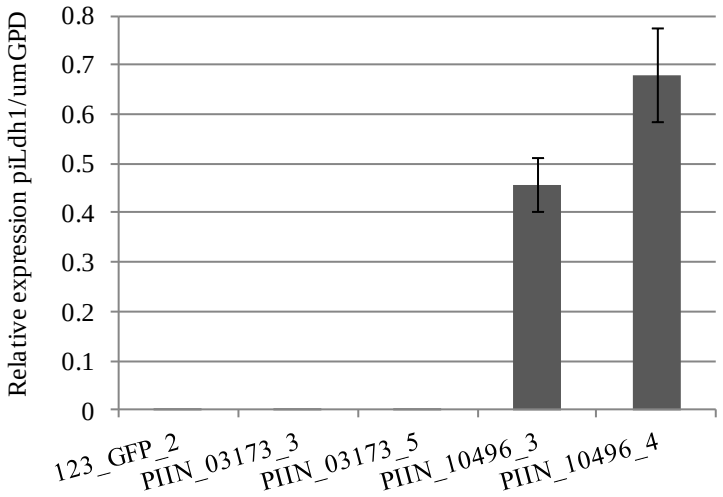

Fig. 42 Expression of *piLdh*1 gene in *U. maydis* transformants. Expression data were normalized using the *umGPD* gene. Error bars represent standard errors of the mean from three biological repetitions.

LC-MS/MS analysis of cell-free culture supernatants after tryptophan feeding was performed to confirm ILA production in the *U. maydis* transformants. Extracts from cell-free culture supernatants of GFP-overexpressing (GFP OE) strains were used as controls. A significantly increased production of ILA was detected only in the *U. maydis* strains expressing *piLdh*1 gene (PIIN_10496, Fig. 43A). Amount of free IAA was slightly decreased in these strains may due to the fact that ILA and IAA use the same precursor (IPA) (Fig. 43B).

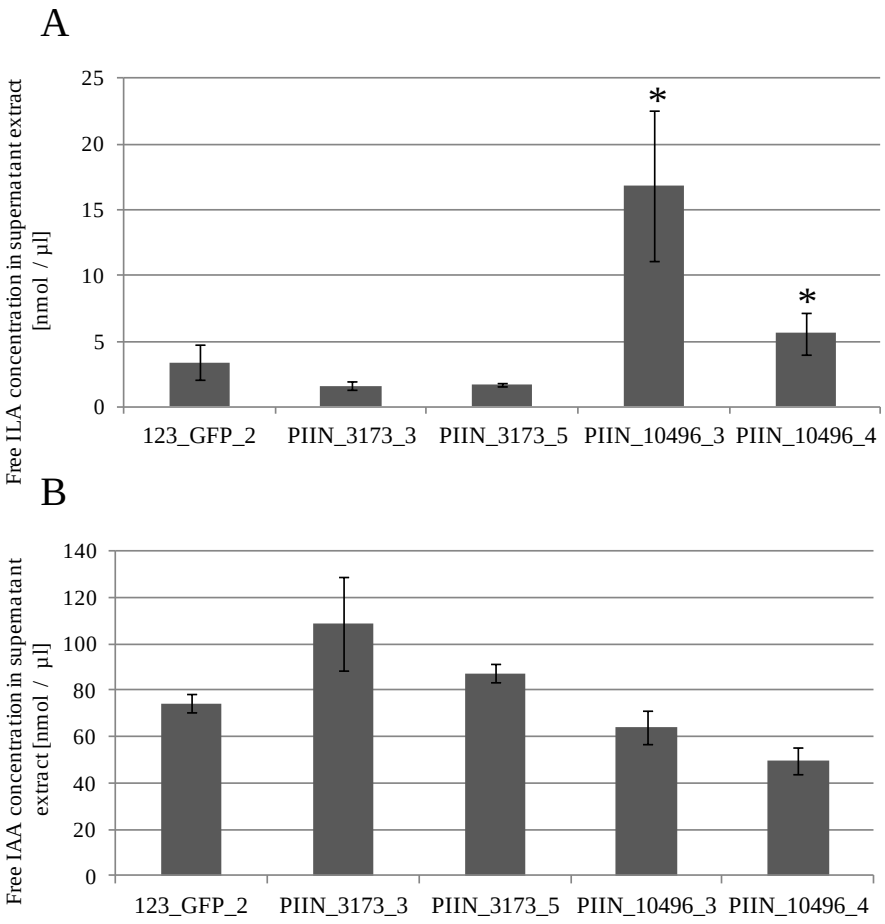

Fig. 43 LC-MS/MS analyses of free ILA and fee IAA levels in *U. maydis* OE strains. A: free ILA and B: free IAA in culture supernatant extracts from *U. maydis* transformants after tryptophan feeding test. GFP OE was used as control. Error bars represent standard errors (n=4). Asterisks indicate significant differences between *U. maydis piLdh*1 overexpressor and the *U. maydis* control transformants ($P<0.05$).

Overexpression of *piLdh*1 did not influence pathogenicity of *U. maydis*. Upon infection with the solopathogenic strain SG200 overexpressing GFP as well as with two independent transformants overexpressing *piLdh*1 40 – 65% of maize plants showed tumor formation (Fig. 44).

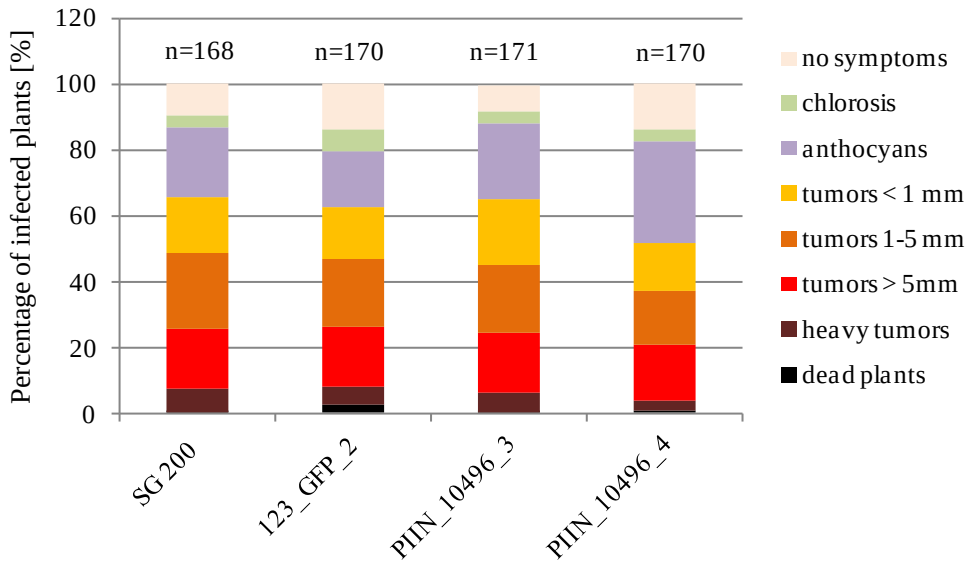

Fig. 44 Pathogenicity assay of *U. maydis* transformants overexpressing *piLdh*1 gene. Numbers on top of the bars represent the number of infected plants. Plants were scored 7 dai (E. Meyer). Strain SG200 and strain with overexpressed GFP gene were used as control.

2.3.3.2. Differences in ILA production in Sebacinales

The ability of *P. indica* to colonize many different host plants and to promote their growth is not a unique trait of *P. indica* but is widely reported in the order Sebacinales. In particular, isolates classified as *Sebacina vermifera* which are closely related to *P. indica* have been reported as growth inducers (Barazani et al., 2005; Desmukh et al., 2006; Ghimire et al., 2009; Baltruschat, personal communication). Since results of this study showed that *P. indica* has the ability to produce not only IAA but also a weak auxin analogue ILA, the facility to consume exogenously supplied tryptophan of three *P. indica* closely related fungi was verified. To the one-week-old cultures of *P. indica*, *Piriformospora williamsii* (Basiewicz et al., 2012), and two *S. vermifera* strains (MAFF305830 and MAFF305842), 2.5 mM tryptophan solution was applied. Culture supernatants extracted 3 dai and separated on a TLC plate clearly showed IAA production in all tested strains (Fig. 45). Only in *Piriformospora* species production of ILA was detected. In *Sebacina* vermifera strains, production of an unidentified violet indole derivative was observed.

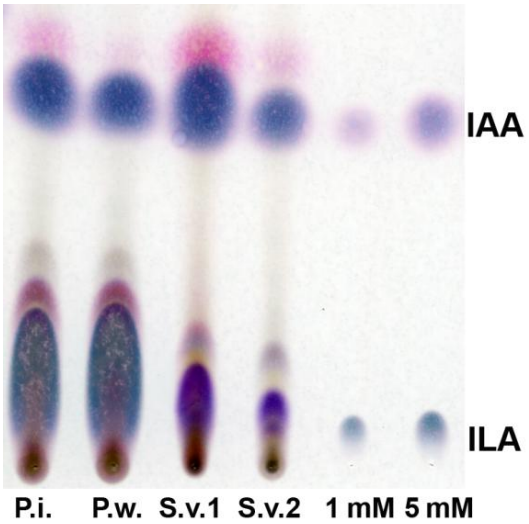

Fig. 45 TLC analysis of differences in auxin production between *P. indica* closely relative Sebacinales species. P.i. – *P. indica*; P.w. – *P. williamsii*; S.v.1 *S. vermifera* (MAFF305830); S.v.2 – *S. vermifera* (MAFF305842). A chloroform:methanol:water (84:14:1)mixture was used as a running buffer.

3. Discussion

3.1. *P. indica* can be genetically modified

Identification, subcellular localization and functional characterization of symbiosis determinants require a diverse array of experimental tools based on genetic engineering. Over the past years many different protocols for genetic transformation have been developed for several major fungal model organisms. The most common methods used are the polyethylene glycol (PEG)-mediated protoplast transformation (Peng et al., 1993; Amey et al., 2002; Kilaru et al., 2009) and *A. tumefaciens*-mediated transformation (ATMT; de Groot et al., 1998; Chen et al., 2000; McClelland et al., 2005), followed by electroporation (Matsuda et al., 1989; Kuo et al., 2004; Kuo & Huang, 2008) and particle bombardment (Bills et al., 1995; Forbes et al., 1998). However, many of the basidiomycete fungi like *Coprinus cinereus* (Binningerl et al., 1987), *U. maydis* (Wang et al., 1988), *L. laccata* (Barrett et al., 1990), *Lentinus edodes* (Sato et al., 1998), *Hebeloma cylindrosporum* (Marmeisse et al., 1992), *Pleurotus ostreatus* (Peng et al., 1993) and *Clitopilus passeckerianus* (Kilaru et al., 2009) are transformed using a PEG-mediated approach. In comparison to ATMT and electroporation, this method requires production and isolation of protoplasts. Protoplasts can be obtained from germinating spores, yeast cells, or active growing mycelia by treating with a mixture of cell wall degrading enzymes such as ß-1,3-glucanases, cellulases, proteases and chitinases. In this study, two different enzyme cocktails were tested for protoplast production in *P. indica*: the lysing enzymes from *T. harzianum* (Sigma) and Novozyme (Novo Nordisc). Use of both mixtures resulted in protoplast formation. In contrast to the results from Peng et al. (1993), who, using three different lysing enzyme mixtures, did not see any significant differences, application of the Novozyme mixture to *P. indica* mycelium resulted in nearly four-fold reduction in the protoplastation time. The age of the culture is one of the crucial factors in efficient protoplasts formation. In *P. ostreatus* protoplast yield was higher using young, actively-growing mycelia (Peng et al., 1993). Use of *P. indica* mycelium, which was crushed and regenerated for 2-3 days, resulted in the highest number of protoplast. From such treated mycelia, approximately 10^7 - 10^9 protoplasts/ml were obtained. Only a small amount of

protoplasts were produced from chlamydospores (approx. 10^5- 10^6 protoplasts/ml) despite longer protoplastation time (up to 3h) with no production of transformants after incubation with different vectors. Fungal chlamydospores provide the genetic link between one generation and another and are often a long-lived survival structures. It is therefore possible that the cell wall composition of chlamydospores differ significantly from that of vegetative mycelia and thus another enzyme cocktail might be needed for hydrolysis.

Storage at -80°C negatively influenced the regeneration ratio of *P. indica* protoplasts and completely inhibited transformation. As it is known that protoplast viability strongly depends on Ca^{2+} concentration (Kuwano et al., 2008) and that the calcium ions may participate in the formation of pores in the cell membrane during uptake of foreign DNA (Olmedo-Monfil et al., 2004), $CaCl_2$ was present in the freezing buffer under the stabilizing influence of glycerol. Nevertheless the applied concentration of calcium ions and glycerol might not be sufficient for efficient protoplast regeneration after freezing and further experiments should be done.

For efficient protoplast regeneration, appropriate osmotic stabilizers are required (Peng et al., 1993; Schuren & Wessels, 1994). We have reported in Zuccaro et al. (2009) that from among three commonly used stabilizers (sucrose, sorbitol and mannitol), the best regeneration of *P. indica* protoplasts occurred in CM supplemented with 0.3 M sucrose. Additionally, exchanging CM with MYP medium resulted in about a two-fold acceleration of regeneration time. Kilaru et al. (2009) reported that incubation of transformed protoplasts for 48 h on agar plates without any selection marker could additionally increase the transformants yield. Besides the regeneration conditions of protoplasts, the presence of homologous promoter regulatory sequence may play a pivotal role in obtaining transformants (Schuren & Wessels, 1994). Although the use of a vector carrying the *Hpt* gene under the homologous GPD promoter did not increase the *A. bisporus* transformation efficiency compared to the heterologous promoter from *A. nidulans* (van de Rhee et al., 1996) nor did Kilaru et al. (2009) observe significant differences between GFP fluorescence intensities driven either by the heterelogous *A. bisporus* GPDII promoter or by the homologous *C. passeckerianus* tubulin promoter, in our laboratory transformation of *P. indica* was successful only when an homologous

promoter was used. Introduction of the two vectors pvv26 and pBShhn-pTEF, both with hygromycin resistance under the control of *P. indica* TEF promoter, resulted in 2% and 1.5% transformation efficiency, respectively. Although the vector pBGgHg carrying the *Hpt* gene driven by *A. bisporus* GPD promoter was efficiently transformed into *L. bicolor* S238N (Kemppainen et al., 2005), *H. cylindrosporum* (Müller et al., 2006) and *C. passeckerianus* (Kilaru et al., 2009), this vector did not work for *P. indica* transformation. On the other hand, it seems that the transformation method used could influence transformation efficiency in *P. indica*. Namely, Yadav et al. (2010) have reported a protocol for electroporation of *P. indica* crushed mycelia using a vector containing the ascomycete *A. nidulans* promoters (pSPiPTD-1G). PEG-mediated transformation attempts in our laboratory using their empty vector (pSilent-Dual1, Nguyen et al., 2008) failed in transformant production. In some fungi, plasmid linearization with the appropriate restriction enzyme has been shown to increase transformation efficiencies (Suzuki et al., 1983; Wang et al., 1988; van de Rhee et al., 1996; Horowitz et al., 2002). Similarly, linearized and purified vectors were more efficiently taken up by *P. indica* and resulted in the regeneration of a larger amount of transformants. *P. indica* transformation could be further enhanced by restriction enzyme mediated integration (REMI, Yi Ding, personal communication). In this method, linearized plasmid DNA is transformed in the presence of a restriction enzyme which generates compatible ends (Kahmann & Basse, 1999). It has been reported by Akamatsu et al. (1997) that the transformation efficiency was raised with the increasing possibility of plasmid integration to the place where the genome has been nicked by the same restriction enzyme. Insertion site, copy number, and orientation can affect expression of the transformed DNA. Confirmation of stable insert integration into the genome of *P. indica* transformants was performed by Southern blot. In contrast to other PEG-mediated transformation reports (Amey et al., 2002; Kilaru et al., 2009), not only multiple-copy but also single-copy insertion could be observed in *P. indica* transformants. Most of the *P. indica* strains with single-copy insertions were transformed with the addition of 10 U/µl of restriction enzyme to the transformation mixture, which was reported to be a highly favourable side effect of the REMI technique (Akamatsu et al., 1997; Kahmann & Basse, 1999). Single-copy insertion is on the one hand desired in order to reduce the possibility of negative effects of

transformation by the accidental destruction of essential genes. On the other hand, repeated copies of the transgene can act multiplicatively and positively affect expression of the transformed DNA. Thus, application of additional restriction enzyme into the transformation mixture should be used cautiously.

3.1.1. GFP reporter system is functional in *P. indica*

The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (Ward, 1979) exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range and it is commonly used as reporter in living cells and organisms (Cubitt et al., 1995). The fluorescence is generated by cyclization and oxidation of the Serine-Tyrosine-Glycine (Ser-Tyr-Gly) sequence at positions 65-67. This oxidation is cell independent and does not require any cofactors or additional enzymes (Heim et al., 1994). Thus, GFP can be used without prior cell lysis and for the fluorescence analyses any additional biochemical treatments like fixation or staining are not needed. As a universal in vivo marker, GFP fusions are frequently used in localization of proteins, protein interactions (Straight et al., 1996), and promoter activity studies (Bowyer et al., 2000; Rohel et al., 2001) as well as for the visualization of whole organisms during plant-microbe interaction studies (Spellig et al., 1996; Maor et al., 1998; Bottin et al., 1999; Huh et al., 2003; Doehlemann et al., 2009). Although GFP is functional in many heterologous systems, some changes have been undertaken in order to optimize and enhance its gene expression, like base modifications for appropriate codon usage, amino acid modification such as serine to threonine shift at amino acid 65 and removal of cryptic intron sites (Cubitt et al., 1995; Chiu et al., 1996; Yang et al., 1996; Stewart, 2001; Poeggeler et al., 2003). During this study, five different vectors were constructed and analyzed in *P. indica* and additionally in *U. maydis*. In the first attempt, GFP sequence from the plant expression vector pGY-1-GFP was cloned under the piTEF promoter resulting in the pTGTh vector. In contrast to transformed barley plants where the green fluorescence reporter gene was strongly expressed and fluorescence was visible under confocal microscope (Eichmann et al., 2003), *P. indica* transformants, verified by Southern and Western blot analyses, did not show any fluorescence. These could be due to the codon usage or to the presence of two copies of the piTEF promoter in this vector which may provoke transcriptional gene silencing through the methylation

and inactivation of the native promoter (Peremarti et al., 2010). With the availability of *P. indica* genome, microarrays were conducted in our group. Analysis of codon usage of the top 100 strongest expressed genes of *P. indica* made it possible to design and order *P. indica* optimized GFP sequence from GenScript (oGFP). The codon optimized GFP under the control of the PiGPD promoter was used for the construction of pGoGFP vector. Additionally two vectors carrying either the uGFP or oGFP fused to *Hpt* were constructed (pTGFPh and pToGFP). Use of these latter vectors resulted in a weak GFP fluorescence for *P. indica* transformants carrying vector pToGFP or in no fluorescence in pTGFPh carrying strain although Southern blot analyses showed accurate plasmid integration into the genome. One possible explanation could be an incorrect folding in *P. indica* cells of the GFP:Hygromycin fusion which resulted in low fluorescence intensity (Wang & Chong, 2002). Western blot analysis of selected transformants showed a band of 27 kDa corresponding to the size of the GFP protein alone. This suggests degradation of the GFP-fusion protein during the protein extraction process or posttranslational changes occurring in *P. indica*. Mistakes in the vector sequences can be excluded as all of the constructed vectors were introduced into *U. maydis* and resulted, although with diverse intensity, in fluorescence activities. While oGFP expressed in another cassette other than *Hpt* gene (pGoGFP) showed clear and strong fluorescence signal. This difference can depend on the strength of the employed promoter. Namely, only in plasmid pGoGFP the GFP gene is driven by piGPD promoter instead of piTEF. Transformants carrying plasmid pMZGFP with GFP under the control of piTEF, showed only slightly weaker fluorescence intensity than transformants carrying the pGoGFP vector. In contrast to other GFP vectors, pMZGFP contains only 600 bp of piTEF promoter and additionally instead of nos- the *P. indica* tef-terminator was used. The use of different terminators can influence gene expression (Platt, 1986; Dean et al., 1989). Accordingly, this data suggests that the use of piGPD promoter and pitef terminator in tandem could result in the strongest fluorescence intensity. As in case of pMZGFP, the use of promoters and terminators which control the same gene enhances the possibility for homologous recombination. Although, no conclusive results to date were produced in our laboratory for the occurrence of homologous recombination in *P. indica,* transformation using pMZGFP did not affect efficiency and the strains carrying this vector did not show any apparent additional

phenotype. In conclusion, introduction of the pGoGFP vector into the *P. indica* genome resulted in the highest green fluorescence and was successfully used for *P. indica* promoter analysis (2.3.1.1) and GFP:fusion protein localization studies (Lahrmann and Zuccaro, unpublished). This provides a basic tool to elucidate subcellular localization of proteins in *P. indica* and during plant interaction.

3.1.2. RNAi – mediated gene silencing in *P. indica*

The fact that *P. indica* hyphae are dikaryotic makes the application of gene knock out methods difficult. Therefore another technique for downregulation of the genes had to be established for this fungus. RNA interference (RNAi) is a conserved eukaryotic posttranscriptional gene silencing (PTGS) method, known as a host defence reaction against viruses and transposable elements. One of the advantages of the application of the RNAi system is its localization. Namely, interaction of the short double-stranded RNA with the catalytic RISC component (argonaute) occurs in the cytoplasm, where all cognate mRNA molecules are available. Therefore the degradation efficiency of mRNA from numerous gene copies and different nuclei in heterokaryotic fungi is higher (Nakayashiki & Nguyen, 2008). Additionally, incomplete inhibition of gene expression might rescue the lethality effect of null mutants and make analysis of intermediate phenotypes in essential biochemical pathways easier. Moreover, this method can be used to silence simultaneously multiple copies of a gene family (Zhao et al., 2005). The components of the RNAi pathway include the RNase III like nuclease dicer, argonaute proteins which are core components of RNA induced silencing complex (RISC) and RNA-dependent RNA polymerases (RdRPs). In some fungi as *Neurospora crassa* and *Schizosaccharomyces pombe*, the presence of these RNAi mechanism components has been described (Galagan et al., 2003; Woolcock et al., 2011) but these elements are not available in all eukaryotes, e.g. in *S. cerevisiae* and *U. maydis* these components are missing (Aravind et al., 2000; Kämper et al., 2006). Bioinformatical analyses of the *P. indica* genome revealed the presence of 4 genes putatively identified as argonaut (PIIN_03690, PIIN_00261, PIIN_05928, PIIN_00081) and 2 genes predicted to be dicers (PIIN_03414, PIIN_02669) and several RdRP like genes (RNA_directed_RNA_polymerase) may involved in the RNA silencing machinery (A. Zuccaro, personal communication). The following are the two main RNAi strategies

used for gene silencing studies in fungi. The first method, described in *Cochliobolus sativus, L. bicolor* and *Schizophyllum commune*, uses a hairpin RNA (hpRNA) construct (de Jong et al., 2006; Kemppainen & Pardo, 2010; Leng et al. 2011). Although it has been reported that this method is more efficient for e.g. *Magnaporthe oryzae* gene silencing, this time consuming and multiple-step cloning strategy is often substituted by a second gene knock down method by using convergent dual promoter systems (Nguyen et al. 2008). To verify the importance of the *P. indica Tam*1 gene in auxin production, this latter system was applied. Thus, two homologous promoters were used (piTEF and piGPD), driving the transcription in opposite directions (Yi Ding and A. Zuccaro). As controls, in order to exclude negative effects produced by the activation of the RNAi machinery and by the presence of convergent homologous promoters, an RNAi empty vector was introduced in *P. indica* and an RNAi strain targeting the ammonium transporter *piAMT*1 was add to the analyses (kindly provided by Yi Ding). The *piTam*1 RNAi strains had all a single-copy insertion and a relatively low silencing efficiency. Although, it has been reported that RNAi silencing does not depend on the number of insertions in *Aspergillus fumigatus* and *Ophiostoma floccosum* (Tanguay et al., 2006; Henry et al., 2007), the presence of a single-copy insertion in *P. indica* transformants can eventually explain the low efficiency of silencing. Van Maerken et al. (2009) showed that the siRNA silencing efficiency might be underestimated depending on the location of the binding sites of primers applied for checking the knocked down gene expression. Thus, additional primer pairs could be tested to verify this thesis in *P. indica piTam*1-transformants. A possible explanation for such low silencing yield might be due to the fact that the size of the employed construct was small (with 131 bp). Additionally, the absence of terminators in the dual promoter system increases the possibility of over reading of the insert with production of off target transcripts which may lead to unintended silencing of different targets. Thus, to prevent over reading of the insert, longer sequence of the targeted gene should be tested. RNA silencing experiments in *M. oryzae* using the vector pSilent-Dual1, have shown that the minimum required insert size is 300-500 bp (Nguyen et al., 2008). Additionally in order to prevent multiple applications of *P. indica* essential gene promoters, examination of activity of *S. vermifera* promoters in the *P. indica* system may be a promising solution.

Henry et al. (2007), observed the presence of morphologically different sectors on the plates of *A. fumigatus* RNAi strains, reverting to the wild type phenotype already after the fist transfer onto new medium. The authors could show excision of at least part of the previously integrated plasmid. This loss of RNAi phenotype was estimated to occur in 50% of the transformants. Similar results have been observed also in *C. sativus*, *Aspergillus parasiticus* and *Trichoderma asperellum* RNAi transformants (McDonald et al., 2005; Ziv & Yarden, 2010; Leng et al., 2011). Even though at much later time points we also observed the reversion of the *P. indica piTam*1 RNAi strains in about 60% of the transformants. The observed revertant phenotype confirmed by biochemical and molecular analyses suggests the excision or inactivation of the inserted plasmid.

3.2. *P. indica* produces auxin in a tryptophan dependent manner

P. indica colonizes the roots of many plant species including mono- and dicots, orchids and mosses. The apparent lack of species specificity suggests that this symbiosis is based on general recognition and signaling processes. This study was aimed to investigate whether auxin, a phytohormone involved in mycorrhizal association and many forms of other plant microbe interactions, is involved in the biotrophic interaction between *P. indica* and barley. In contrast to the broad insight into IAA biosynthesis pathways in plants and bacteria (Spaepen et al., 2007) little is known about IAA biosynthesis in fungi. The common pathway used by fungi for IAA production is IPA-mediated and is known to be tryptophan dependent (Robinson et al., 1998; Chung et al., 2003; Chung & Tzeng, 2004; Reineke et al., 2008). One of the oldest methods, still in use, to measure auxin and its derivatives in the culture supernatant of fungi is the colorimetric reaction using Salkowski reagent. The colour reaction which varies from pink to red and yellow depends on the presence of oxidized IAA and its derivatives and on the amount of these compounds in the oxidation reaction mixture (Meudt & Gaines, 1967). More accurately, the colour derives from the oxidized indole ring and depends on the type of substitution. Thus, the Salkowski reagent can be used only for verifying the presence of indole derivatives but it is not specific. Sirrenberg et al. (2007) have applied this reagent for the identification of indoles in *P. indica* culture supernatant. Additionally they measured 1.55 µM +/- 0.2 IAA by GC-MS in 4 weeks old *P. indica*

liquid cultures. Although no additional application of exogenously tryptophan was applied in their study, the used medium contained complex nutrients such as yeast and malt extract and it could be speculated that enough tryptophan was available for fungal auxin production. Along the same lines, in this study very small amounts of IAA were observed during growth of *P. indica* in liquid CM without additional tryptophan application. No auxin was detected during cultivation of *P. indica* in different minimal media without tryptophan, so that we could not verify the presence of a tryptophan independent pathway for *P. indica* auxin production. The different indole derivatives can additionally be identify by separation using TLC, often combined with High-Performance Liquid Chromatography (HPLC), Gas Chromatography–Mass Spectrometry (GC-MS) or LC-MS/MS (Tanaka et al., 2003; Chung et al., 2003; Somers et al., 2005; Reineke et al., 2008; Contreras-Cornejo et al., 2009). The presence of auxin in a given solution can also be confirmed by its biological activity. Here, two methods are often used: the coleoptile elongation test and the dose-response cotyledon test (Nitsch & Nitsch, 1956; Park et al., 2001; Mattsson et al., 2003). During this study, *P. indica's* indole derivatives production has been analysed by TLC, LC-MS/MS, coleoptile elongation test and dose-response cotyledon test. The only pathway detected in *P. indica* during this study is via the IPA route. In some microbes the presence of diverse pathways for IAA production is proposed to correlates with different functions. For example, in *Erwinia herbivola* pv. *gypsophilae* (*Ehg*), evidence for IPA- and IAM-mediated pathway has been reported (Manulis et al., 1998). An insertional mutagenesis of the IPA decarboxylase gene (*ipdC*) in this gall-formed bacterium resulted in inactivation of the IPA-pathway. The survival of these bacteria mutants on bean plants was significantly lower than that of wild type strains and strains with a mutated IAM-pathway. Additionally, a lack of the functional IAM-mediated pathway in the mutant strain impaired pathogenicity, which was visible by a significant decreased size of galls. Similar results were obtained by Hsu (2010) in *Streptomyces scabies,* showing that mutations in genes involved in the IAM-mediated pathway significantly reduced bacterial IAA production as well as inhibited its virulence. A study by Reineke et al. (2008) presenting deletion mutants of IPA-mediated pathway in *U. maydis* indicated a non significant role of this pathway on tumor formation, even though the level of IAA *in planta* was lower than in wild type infected plants. In order to analyze the role of *P.*

indica auxin production during plant colonization, genes putatively involved in the IPA-mediated pathway were further characterized.

3.2.1. *piTam*1 gene is a key player in IAA production

The IPA-mediated pathway consists of three enzymatically controlled steps. In the first step, conversion of tryptophan to IPA is catalyzed by aromatic-amino-acid transaminases (AAT). In *S. cerevisiae*, two genes, *ARO8* and *ARO9*, encoding aromatic-amino-acid transaminases were found (Iraqui et al., 1998). *ARO8* was described to be constitutively expressed and to be responsible for phenylalanine and tyrosine biosynthesis, whereas *ARO9* was mainly involved in tryptophan degradation (Iraquai et al., 1998). Both genes are involved in IAA production and their mutations not only decreased the level of tryptophan-derived IAA but also impaired invasive growth and the formation of pseudohyphae (Rao et al., 2010). In *U. maydis* two aromatic-amino-acid transaminases, *Tam1* and *Tam2*, have also been identified (Reineke et al., 2008). Analyses of single (*Δtam1, Δtam2*) and double mutants (*Δtam1Δtam2*) proved both of these genes to function as tryptophan aminotransferase, where double mutants produced lower amount of IPA from tryptophan than both separately analyzed single mutants. The activity of AAT proteins in cell-free crude extract from the N_2-fixing bacteria *Azospirillum sp*. was reported by Pedraza et al. (2004) and these were proved to be involved in conversion of tryptophan into IPA. In *P. indica* three genes were predicted to encode proteins which might be involved in transamination of tryptophan. Blastp analyses have shown that the PIIN_07534 (*piTam*1) gene has a 43% identity to the *U. maydis Tam1* gene, the PIIN_08984 (*piAro*8) gene possesses a 31% identity to *S. cerevisiae ARO8* and the PIIN_01989 (*piAro*9) gene has 25% identities to *S. cerevisiae ARO9*. Expression analysis after tryptophan treatment revealed that PIIN_07534 is tryptophan responsive whereas *piAro*8 and *piAro*9 are constitutively expressed. As reported for *S. cerevisiae*, some AATs might be involved in transamination of aromatic amino acids other than tryptophan. Further testing using other aromatic precursors may provide conclusive results. Additionally, another medium could be tested to exclude inhibitory effect of CM ingredients on gene expression of AATs. Dependence of the *piTam*1 gene expression on tryptophan has been additionally verified using a GFP reporter system by fusing the GFP gene sequence with 230 bp sequence upstream of the

*piTam*1 gene. The intensity of green fluorescence correlated with the concentration of tryptophan in the media and was also detected in hyphae during the early colonization of barley roots. These results strongly indicate that the *piTam*1 gene is expressed during establishment of biotrophy and that plant tryptophan is available in this early colonization stage in roots. As AAT are involved in the first step of IAA production, its downregulation should significantly influence the amount of formed auxin. Analysis of RNAi strains carrying a silencing construct of 131 bp of the *piTam*1 gene sequence resulted in up to 98% reduction of IAA production upon tryptophan feeding compared to the empty vector controls, visualized on TLC plate and verified by LC-MS/MS. Zuther et al. (2008) showed that the knock out of the *Tam1* and *Tam2* genes in *U. maydis* resulted in significantly weaker production of indole-derived pigments on medium supplemented with tryptophan. In contrast, *P. indica* transformants with a silenced *piTam*1 gene produced pink-brownish pigments after tryptophan feeding. Pigments production was not observed in wild type and empty vector controls. Research by Zuther et al. (2008) has shown that *U. maydis* and *Malassezia furfur* pigments are a consequence of spontaneous conversion of IPA. *PiTam*1 silencing could have induced other tryptophan dependent which lead to pigment formation. Further analyses should be undertaken in order to clarify the origin of *P. indica* transformant's derived pigments. Furthermore, silencing of the *piTam*1 gene resulted in a less compact colony growth phenotype. These phenotype changes can be partially complemented by addition of exogenous auxin to the culture. Similarly, Rao et al. (2010) observed the same dependence of phenotype to IAA in *S. cerevisiae* mutants impaired in auxin production. Knock out of genes involved in the conversion of IAD into IAA resulted in enhanced filamentation compared to the wild type strain. Application of exogenous IAA partially rescued the wild type phenotype.

3.2.2. Role of *piAdh* genes in *P. indica* IAA production

The final step in IAA production in the IPA-mediated pathway is controlled by aldehyde dehydrogenases (Adh). These enzymes are involved in the oxidation of aldehyde group into the carboxylic acids group. These enzymes, in the presence of NAD^+, can catalyze the conversion of IAD into IAA. In *U. maydis* acetaldehyde dehydrogenases (ladl and Iad2) have been analyzed biochemically and molecularly

(Basse et al., 1996) in culture and also during the interaction with maize plant (Basse et al., 1996; Reineke et al., 2008). Furthermore, in *S. cerevisiae* two genes putatively involved in IAD oxidation have been identified *(Ald2* and *Ald3)* and showed 50% (*Ald3*) and 49% (*Ald2*) protein sequence identity with *U. maydis Iad1*(Rao et al., 2010). Though constructed deletion mutants produced, in comparison to the wild type strain, lower amount of radioactive IAA-derived from radioactive tryptophan, *Ald2* and *Ald3* were not required for IAA-induced filamentation in yeast. In *P. indica* genome two genes (*piAdh*1 and *piAdh*2) have been predicted to function in the IAD-IAA conversion step. Interestingly, the carbon source used for *U. maydis* experiments drastically changed the expression of *Iad* genes in this system. The enzyme activity in IAA formation from IAD of *ΔIad1* mutant was drastically decreased in CM containing glucose. Additionally, change of substrate affinity has been observed during this condition and TOL was produced instead of IAA. The enzyme activity of Iad2 was also NAD^+-dependent but only in medium containing arabinose, while in glucose containing medium this activity was strongly inhibited. In an *Iad1*-null mutant cultivated in CM supplemented with arabinose and TAM, IAA production could be observed, strongly indicating a role of *Iad2* in TAM-IAD-IAA conversion step. Nevertheless, application of glucose to this culture inhibited completely IAA production (Basse et al.. 1996). The double mutants Δiad1Δiad2 were not able to convert TAM into IAA (Reineke et al., 2008), demonstrating function of these enzymes in auxin production in *U. maydis*. Based on these results, experiments with different carbon sources have been performed with *P. indica.* Gene expression was examined and showed opposite regulation of the *piAdh* genes. The *piAdh*1 was upregulated in glucose containing medium and CM supplemented with tryptophan, whereas *piAdh*2 was upregulated in arabinose containing medium.

Cultivation of *P. indica* wild type strain in CM supplemented with IAD resulted in TOL but not IAA production, whereas in minimal medium TOL as well as IAA were efficiently produced. In contrast to the *P. indica* wild type strain, feeding tests experiments with *piTam*1 RNAi strains in CM showed a high amount of TOL and IAA. Silencing of *piTam*1 may results in derepression of *piAdh* genes expression as suggested from preliminary data in our lab (data not shown) and therefore in the production of IAA from IAD in CM medium.

3.2.3. ILA has a weak auxin activity

Cultivation of *P. indica* in medium supplemented with tryptophan resulted in the accumulation of 3 fold more ILA than IAA. ILA production or/and utilization have been reported for *C. acutatum*, *C. gloeosporioides* and *Aciculosporium take* (Robinson et al., 1998; Tanaka et al., 2003; Shilts et al., 2005), and additionally in *Ustilago esculenta*, where it was used as an efficient substrate for IAA production (Chung & Tzeng, 2004). Up to now, no reports on fungal genes involved in ILA production have been published. Expression of the gene5 *in planta* after integration of the Tplasmid from *A. tumefaciens* resulted in ILA accumulation after tryptophan treatment (Körber et al., 1991). Trinchant and Rigaud (1974) biochemically analyzed ILA production in the bacterium *R. meliloti*. The LDH-EC 1.1.1.27 was proven to be a soluble lactate dehydrogenase involved in interconversion of pyruvate into lactate. *P. indica* is known to be associated with the endobacterium *R. radiobacter* (Sharma et al., 2008). Symbiosis with endobacteria raises the possibility of horizontal gene transfer. Additionally, ILA production may be the result of bacterial activity. In this study it was shown that *R. radiobacter* is not able to produce ILA from tryptophan suggesting that ILA derived from *P. indica* produced enzymes. Genome screening of *P. indica* revealed 7 candidate genes, where 6 of them were classified to be closely related to lactate dehydrogenases from other fungi and only one, PIIN_10496 (*piLdh*1*)*, displayed homology to bacterial LDH. Additionally a second gene encoding D-lactate dehydrogenase was analysed PIIN_03173. PIIN_10496 gene was tryptophan responsive, whereas PIIN_03173 was not. To confirm functionality of the *piLdh*1 gene, it was overexpressed in *U. maydis*. LC-MS/MS analyses proved enhanced ILA accumulation in *U. maydis* transformants expressing *piLdh*1. Körber et al. (1991) showed that transgenic tobacco seedlings overexpressing gene5 did not show any differences in the ability of organ differentiation. However, application of ILA to undifferentiated callus tissue resulted in shoot formation, suggesting hormonal imbalance between cytokinin and auxin for cytokinin benefits. These authors proposed that ILA might be an auxin antagonist competing with IAA against protein involved in auxin transport and signaling. In contrast, Sprunck et al. (1995) compared the effect of different auxin, including ILA, on *Pisum sativum* stem elongation, callus and root

formation. They did not observe antagonistic correlation by subsequent treatment of auxin analogue NAA with ILA. Binding experiment using the auxin binding protein ABP44, these authors stated that ILA is a weak auxin analogue like NAA rather than an IAA antagonist. Interestingly, Körber et al. (1991) highlighted that the proposed auxin antagonistic effects of ILA may depend on type and differentiation stage of the target plant tissues. Gibson et al. (1972) showed that different plants possess different abilities to produce and utilize ILA. This could explain the differences in the experiments of Körber et al. (1991) and Sprunck et al. (1995). It could also be the reason why, during this study, weak auxin activity of ILA could be determined in tomato cotyledons. Tomato plants were shown to be able to use ILA (Gibson et al., 1987). Interestingly, analyses of the *P. indica* closely related species *S. vermifera* and *P. williamsii*, showed that only *P. williamsii* is able to produce ILA in large amount after tryptophan feeding. Two *Sebacina* strains synthesized large amounts of IAA but ILA production could not be detected under the tested conditions. As *P. indica* produced both of these indole components and is known to colonize many different plants, it could be speculated that ILA might be involved in broadening the host spectrum and/or beneficial effects. Studies using *piLdh*1 silenced *P. indica* transformants and *piLdh*1 overexpressor *S. vermifera* strains could help clarifying the role played by ILA in the interaction with hosts.

In the fungus *P. tinctorius* an endogenous auxin regulatory system has been described which was activated during the early plant-microbe interaction. Besides IAA production, accumulation and releasing of an auxin antagonist, a tryptophan betaine called hypaphorine, was identified (Nehls et al., 1998; Ditengou & Lapeyrie, 2000; Ditengou et al., 2000; Reboutier et al., 2002; Ditengou et al., 2003; Dauphin et al., 2007). The amount of this indolic compound increased during colonization of eucalyptus seedlings and its level was higher than IAA. Additionally, it antagonizing effect on exogenous IAA activity on eucalyptus root elongation has been reported (Ditengou & Lapeyrie, 2000). Hypaphorine has not been identified in all ectomycorrhizal fungi (Felten et al., 2012). It is possible that other indole derivatives may play an important role in auxin antagonism regulating ectomycorrhizal development. According to this data, it is probable that *P. indica* may produce a large

amount of ILA in order to control endogenous auxin production as well as for manipulation of auxin responses in different host plants.

3.3. Role of IAA in the establishment of biotrophy

Production of auxin has been described in many plant associated microbes and hints that IAA of microbial origin may influence host auxin homeostasis has been reported (Yamada, 1993; Llorente et al., 2008; Kazan & Manners, 2009). Chen et al. (2007) showed that the biotrophic plant pathogen *P. syringae* modulates auxin physiology of Arabidopsis plants in order to enhance host susceptibility. Fungal-derived IAA is speculated to influence host cell wall structure and enhance susceptibility to fungal infection (Gogala, 1991). Moreover, Tanaka et al. (2011) studied auxin production in the rice blast fungus *M. oryzae* and were able to show local production of IAA in infecting hyphae during the biotrophic phase.

Exogenously added IAA (1 µM and 10 µM) significantly enhanced *P. indica* colonization of barley roots. *PiTam*1 expression during early interaction with barley roots was induced. Colonization study of the *piTam*1 RNAi strain indicated that impairment of auxin production has a strong influence on *P. indica* colonization during early interaction with barley or on the ability to establish biotrophy. It could be suggested that auxin is involved in germination/sporulation and that the inhibited colonization ratio might be influenced by slower germination and chlamydospore formation of the RNAi transformants. Nevertheless, the amount of chlamydospores collected from the RNAi strain for colonization studies did not significantly differ to wild type and empty vector controls. During axenic cultivation the less compact colony growth phenotype of the RNAi transformants could be partially rescued by exogenously applied auxin. Similar effects could take place during the later time points of *P. indica*-barley interaction, suggesting that host auxin may complement *P. indica* RNAi strains phenotype. LC-MS/MS analyses of the IAA content in colonized and non colonized *P. indica* barley plants showed significant differences in auxin concentration at 3 dai but not at later time points (5, and 14 dai). The amount of measured free IAA is probably too high to be a sole effect of fungal IAA, suggesting that the colonization process influenced the host IAA production and probably transport. This is congruent with

microarray data from 3 dai *P. indica* colonized barley roots, where genes involved in host IAA signaling and production were upregulated (Schäfer et al., 2009). These results would indicate an accumulation of plant IAA during the biotrophic phase. This is in agreement with Kazan & Manners (2009), who stated that during initial plant-microbe interactions most of the IAA is supplied by the host.

Gibson et al. (1972) reported that barley plants, in contrast to tomato plants, cannot produce ILA and in addition are not able to use ILA as substrate for IAA synthesis. In the tested barley strain (Golden Promise), small amounts of ILA were observed, but in comparison to the measured free IAA concentration this was 15-30 fold lower. During the *P. indica*-barley interaction, accumulation of ILA has been detected and its concentration was significantly higher than in mock treated roots. This suggests that the ILA may be of fungal origin and thus strongly indicates an activation of fungal auxin biosynthesis pathway during interaction with roots.

It was reported that microbial auxin production can be involved in growth promotion effects and changes in root morphology (Nassar et al., 2005). During this study the *P. indica* RNAi strains impaired in auxin production were tested for enhanced growth of barley. Surprisingly, no differences between RNAi strains, empty vector controls and wild type strain could be observed. The measured length of the leaves and the dry weight of barley roots inoculated with the Tam2_2 strain were 20% and 139% larger respectively when compared to mock treated control, indicating that the fungal-derived auxin are not involved in triggering beneficial effects to this host. To conclude, the *piTam*1 gene was proven to be involved in auxin production in *P. indica,* to be induced during interaction with barley roots and to play a crucial role in the early colonization process. However, expression of *piTam*1 gene is not required for growth promotion triggered by *P. indica* in barley plants.

4. Material and methods

4.1. Materials and source of supplies

During this Ph.D. study chemicals from the following companies were used: Difco; (Augsburg), Fluka (Buchs; Switzerland), Merck (Darmstadt), Roth (Karlsruhe) and Sigma-Aldrich (Deisenhofen). PROMEGA Wizard® SV Gel and PCR Clean-Up System or QIAquick Gel Extraction Kit was used for cleaning DNA after PCR reaction or digestion. For extraction of *E. coli* plasmids DNA QIAprep® Spin Miniprep Kit and QIAGEN® Plasmid Midi Kit (QIAGEN; Hilden) were used. Other chemicals are described together with certain methods.

4.1.1. Oligonucleotides

The oligonucleotides that were used were ordered and synthesized by MWG Operon or Sigma-Aldrich and are listed in the Table below.

Table 2 Oligonucleotides used during this study.

Primer name	Sequence 5'→3'	Experiment
M13 (-21) For	TGTAAAACGACGGCCAGT	Standard sequencing/colony PCR
M13 Rev	CAGGAAACAGCTATGAC	Standard sequencing/colony PCR
T7 promoter F	TAATACGACTCACTATAGGG	Standard sequencing/colony PCR
03008_QPCR_F	GCAAGTTCTCCGAGCTCATC	Real time qPCR (piTEF)
03008_QPCR_R	CCAAGTGGTGGGTACTCGTT	Real time qPCR (piTEF)
HvUBI_F	CAGTAGTGGCGGTCGAAGTG	Real time qPCR (UBI)
HvUBI_R	ACCCTCGCCGACTACAACAT	Real time qPCR (UBI)
HV_PR1b_for	GGACTACGACTACGGCTCCA	Real time qPCR
HV_PR1b_rev	GGCTCGTAGTTGCAGGTGAT	Real time qPCR
HV_PR10_for	GGAGGGCGACAAGGTAAGTG	Real time qPCR
HV_PR10_rev	CGTCCAGCCTCTCGTACTCT	Real time qPCR

PIIN_07534_qpcr_F	AAAGAAAAGGCGCTCGAAG	Real time qPCR (*piTam*1)
PIIN_07534_qpcr_R	CTCAGCCCCTCGTCTACATC	Real time qPCR (*piTam*1)
PIIN_08984_qpcr_F	TACAAGCACAGCGAATCCAA	Real time qPCR (*piAro*8)
PIIN_08984_qpcr_R	GAACATCCATCCAGGGAGAA	Real time qPCR (*piAro*8)
PIIN_01989_qpcr_F	AGGCTGGGTTGCTGATTCTA	Real time qPCR (*piAro9*)
PIIN_01989_qpcr_R	GAAGCGTCTTGGCAAAAATC	Real time qPCR (*piAro*9)
PIIN_10496_qpcr_F	CCGTGATGGAGAAGAATGGT	Real time qPCR (*piLdh*1)
PIIN_10496_qpcr_R	TCGTTCCATCCAATAGCACA	Real time qPCR (*piLdh*1)
PIIN_03173_F	CCAGGACGATCAACTTAGCC	Real time qPCR
PIIN_03173_R	AAAGAGCGCAGATTCGAGAG	Real time qPCR
PIIN_02674_qpcr_F	CAGAGTCCGTTTGGAGGGTA	Real time qPCR (*piAdh*1)
PIIN_02674_qpcr_R	CTCTCCGACATTCCACAACA	Real time qPCR (*piAdh*1)
PIIN_04899_qpcr_F	GATGTATGGACTGGCTGCTG	Real time qPCR (*piAdh2*)
PIIN_04899_qpcr_R	TGGTAGCTGTGGGTTGAGC	Real time qPCR (*piAdh2*)
PGPDf	GTCGAAGTAAAGGACGGCAA	piGPD promoter analyses
PGPDr	TGACGGCCAAGATTTCAATC	piGPD promoter analyses
GPD383f	CGTATCGTCTTCAGGAATGC	piGPD promoter analyses
GPD383r	TGTGGGTCGTACTTGTCGAG	piGPD promoter analyses
GPDH189f	CTCGAGAAACCTTGACTCC	piGPD promoter analyses
GPDHendr	GGTACAATGACTGTATTGC	piGPD promoter analyses
pGPDHr	TGAGCCCAGAGGGTTACATC	piGPD promoter analyses
pGPDH180r	ATGTGGTGGAAGGAGTCAAGG	piGPD promoter analyses
Gpd1Fshort	GTTGCCGAGGAAGCTGACC	piGPD promoter analyses

gpd-f	GATTGAAATCTTGGCCGTCA	piGPD promoter analyses
gpd-r	TTGCCGTCCTTTACTTCGAC	piGPD promoter analyses
pGPD383f	CTCGACAAGTACGACCCACA	piGPD promoter analyses
pGPD383r	GCATTCCTGAAGACGATACG	piGPD promoter analyses
prom7534_Apa_F	GCGGGCCCCTCGACGTTGCCGGATCCAA	piTAM promoter analyses
prom7534_hindIII_R	CGCAAGCTTTTTACAAAGCTGGATGGGTG	piTAM promoter analyses
HygF-HindIII	CCCAAGCTTATGGAAAAGCCTGAACTCAC	Cloning/colony PCR
HygR-EcoRI	GGAATTCCCTATTCCTTTGCCCTCGGAC	Cloning/colony PCR
HygMid_R	TGCACGGCGGGAGATGCAAT	Cloning/colony PCR/Southern blot
Hygro_F	CGTGCTTTCAGCTTCGATGTAGG	Cloning/colony PCR/Southern blot
Hygro_R	AAGATGTTGGCGACCTCGTATTG	Cloning/colony PCR
ustiGFP_HindIII_F	GCAAGCTTATGGTGAGCAAGGGCGAGGAG	Cloning/colony PCR
Ustinos-SbfI_R	GCCCTGCAGGCTCATGTTTGACAGCTTATC	Cloning/colony PCR
oGFP_NheI_F	GCGCTAGCATGGTCTCCAAGGGCGAGGA	Cloning/colony PCR
oGFP_XmaI_R	GCCCCGGGCTTGTAGAGCTCGTCCATAC	Cloning/colony PCR
Gogfp_EcoRV_R	GCGATATCTTACTTGTAGAGCTCGTCCA	Cloning/colony PCR
Gogfp_EcoRV_F	GCGATATCATGGTCTCCAAGGGCGAGGA	Cloning/colony PCR
EcoRI_oGFP_F	CGGAATTCATGGTCTCCAAGGGCGAGGA	Cloning/colony PCR
EcoRI_oGFP_R	CGGAATTCGTAGAGCTCGTCCATACCGA	Cloning/colony PCR
pTEFend	GAGGGTGGATGTTTGCTCGAGTCG	Cloning/colony PCR
Trans-TEF_F	AACCTTGCCCTCCACCATCAG	Cloning/colony PCR
TEFprom_begR	CAGAGGAACCGATGCTGAAT	Cloning/colony PCR
pGPD_for	ACGACCTCATTGCCTACGAC	Cloning/colony PCR

pGPD_rev	GTTTCTCGAGTTGGGCGTAA	Cloning/colony PCR
nosT-NotI	ATAAGAATGCGGCCGCTAGATCGTTCAAACATTTGGC	Cloning/colony PCR
nosT-SacI	CGAGCTCGGTTTGACAGCTTATCATCG	Cloning/colony PCR
pTEFfusion_f	TCGAGCTCGGTACCCAGACCCCATAGAAGCGCGCC	Cloning/colony PCR
pTEFfusion_r	GCCCTTGCTCACCATTTTTTAGTATGAAAAAGATG	Cloning/colony PCR
GFPfusion_f	TTTTCATACTAAAAAATGGTGAGCAAGGGCGAGGA	Cloning/colony PCR
GFPfusion_r	TCGGTTCGTATTCGTTTACTTGTACAGCTCGTCCA	Cloning/colony PCR
tTEFfusion_f	GAGCTGTACAAGTAAACGAATACGAACCGAGACGC	Cloning/colony PCR
tTEFfusion_r	CTCTAGAGGATCCCCACAATCTCCTTGTCTGGCTC	Cloning/colony PCR
TAM7534_SpeI_F	GCCACTAGTAAAGAAAAGGCGCTCGAAG	RNAi construction
TAM7534_SpeI_R	GCCACTAGTCTCAGCCCCTCGTCTACATC	RNAi construction
10496OVER_BAMHI _F	GCGGATCCATGTCCTCCACGCCAATGGT	Overexpression in *U. maydis*
10496OVER_Not_R	GCGCGGCCGCTTACTCGTCCGTGACCTTGA	Overexpression in *U. maydis*
3173OVER_bamHI_F	GCGGATCCATGCAGATCGCCTTCTTCAG	Overexpression in *U. maydis*
3173OVER_ Not I_R	GCGCGGCCGCTTATTTGGAAGACTTGACAA	Overexpression in *U. maydis*

4.1.2. Vectors

All vectors contain ampicillin or kanamycin resistance cassette for selection of transformed *E. coli*. *F*or selection of *P. indica* transformants hygromycin resistance cassette and for selection of *U. maydis* transformants hygromycin or carboxin resistance cassette was introduced. Cloning steps were prepared with the restriction enzymes Antarctic Phosphatase and T4-DNA ligase from New England Biolabs (NEB; Frankfurt) and Top10 or DH5α *E. coli* strains. All PCR reactions were performed using a proofreading polymerase (Pfu; Promega). Each cloning step during vector construction was first confirmed by colony PCR (Taq polymerase), then through control digestion and finally confirmed by sequencing (MWG Operon).

4.1.2.1. Vectors for TA cloning of PCR products

pGEMT-Easy (Promega, Mannheim) - Vector used for cloning of PCR products. Vector is commercially prepared by cutting with *EcoR*V and adding a 3´ terminal thymidine to both ends. Inserted DNA segments can be cut out with *EcoR*V. The plasmid can be used for the blue-white selection. The sequencing of the insert is possible using T7 and SP6 primers, located on both side near to the *EcoR*V restriction site. Vector possesses ampicillin resistance cassette as selection marker for *E. coli*.

pCRII-TOPO (Invitrogen, Karlsruhe) - Vector used for cloning of PCR products. Topoisomerase activity and overhanging thymidine residues increased cloning efficiency. Inserted DNA segments can be cut out with *EcoR*I. The Plasmid can be used for the blue-white selection. The verification of the inserts is possible by sequencing using M13 and M13Rev primers. Vector possesses ampicillin and kanamycin resistance cassettes as selection markers for *E. coli*.

4.1.2.2. *P. indica* transformation vectors

Starting vectors:

pBShhn-pTEF – vector based on plasmid pBS-hhn (Kämper et al., 2004) contains the hygromycin phosphotransferase gene (*Hpt*) fused to the nos terminator. Hsp70 promoter is exchanged with 1650 bp of *P. indica* TEF promoter. Vector possesses ampicillin resistance cassette as selection marker for *E. coli*. (Zuccaro et al., 2009)

pvv26 - binary vector (kindly provided by Jörg Kämper, Karlsruhe, unpublished) constructed on the backbone of the pPK2-Hyg (Covert et al., 2001) using the restriction sites *Nco*I/*Sac*I in a three fragment ligation. This vector contains the right and left border of the T-DNA for use in *Agrobacterium*-mediated transformation, and *Hpt* gene under control of the piTEF promoter. Vector possesses kanamycin resistance cassette as selection marker for *E. coli*. (Zuccaro et al., 2009)

pBGgHg - vector contains two gene sequences: eGFP and *Hpt* which are

controlled by *A. bisporus* GPD promoter and transcriptional terminator of the cauliflower mosaic virus 35S gene. Both cassettes are located between the left and right border regions of T-DNA from *A. tumefaciens* Ti plasmid. Vector contains kanamycin resistance cassette (Chen et al., 2000).

pAN7-1 – vector contains *Hpt* gene controlled by the *A. nidulans* gpdA and trpC expression signals (Punt et al., 1987). Plasmid possesses ampicillin resistance cassette as selection marker for *E. coli.*

pGY1-GFP – plasmid based on plant expression vector pGY1 (Schweizer et al., 1999) that contains a 540-bp fragment of the CaMV 35S promoter and the CaMV 35S terminator separated by a multiple cloning site and cloned *GFP* gene sequence via *BamH*I restriction site. Vector possesses ampicillin resistance cassette as selection marker for *E. coli.*

pSilent-Dual1 – vector for RNAi silencing in *M. oryzae* (Nguyen et al., 2008) contains two convergent opposing RNA polymerase II promoters: PtrpC and Pgpd from *A. nidulans*. Plasmid possesses ampicillin resistance cassette as selection marker for *E. coli* and geneticin resistance cassette for selection in *M. oryzae.*

pPiRNAi - vector for RNAi silencing. *P. indica* GPD and TEF promoters were used for construction of convergent dual promoter system and cloned in a vector harbouring the hygromycin B resistance gene cassette and separated with multicloning site. Vector possesses ampicillin resistance cassette as selection marker for *E. coli.* Plasmid was constructed by Yi Ding, MPI Marburg, Germany.

Vectors constructed during this study:

pTGTh - The sequences of GFP gene and CaMV terminator were cut from the plasmid pGY1-GFP using *Spe*I and *Sac*I (Fermentas) and subsequently cloned into pGEMTeasy with previously inserted *P. indica* TEF promoter. The whole pTEF::GFP-tCaMV cassette was integrated into the pBShhn-pTEF vector previously linearized with *Kpn*I. Vector contains ampicillin resistance cassette as selection marker for *E. coli.*

pMZGFP - Vector was constructed using a PCR fusion strategy with overlapping primers for *P. indica* TEF promoter, the eGFP sequence from plasmid p123 (uGFP) and *P. indica* TEF terminator. The PCR fragment was inserted into the pBShhn-pTEF vector previously linearized with *Kpn*I (Fermentas). Vector contains ampicillin resistance cassette as selection marker for *E. coli.*

pTGFPh - The vector contains uGFP sequence fused to *Hpt* gene. The uGFP sequence was amplified by PCR and inserted into the pBShhn-pTEF vector using the *NheI* and *Sma*I restriction sites. Vector possesses ampicillin resistance cassette as selection marker for *E. coli.*

pToGFP – The vector contain oGFP sequence fused to the *Hpt*. The uGFP sequence was exchanged with oGFP (*P. indica* codon optimized GFP gene, synthesized by GenScript) using *Nhe*I and *Sma*I (NEB). Vector contains ampicillin resistance cassette as selection marker for *E. coli.*

pGOGFP - *P. indica* GPD promoter sequence was amplified and inserted into the pGEMTEasy vector. The nos terminator sequence was introduced with *NotI* and *Sac*I (NEB) and the oGFP sequence was cloned upstream of the nos terminator with *EcoR*V (NEB). The pGPD::oGFP-tnos cassette was transferred into the pBShhn-pTEF vector using *Kpn*I (NEB). Vector contains ampicillin resistance cassette as selection marker for *E. coli.*

pRNAi7534 – Plasmid constructed for RNAi analyses for *piTam*1 gene. The 131 bp long fragment of the gene PIIN_07534 was amplified by PCR and cloned between the piTEF and piGPD promoters with *EcoR*V. Vector contains hygromycin resistance for selection in *P. indica* and ampicillin resistance for selection in *E. coli.*

pToGFP –Vector used for promoter analyses of *piTam*1 gene. The piGPD promoter sequence was cut out with *Apa*I and *Hind*III (NEB) from the pGoGFP backbone and exchanged with 230 bp of the promoter region upstream of the *piTam*1 gene. Vector contains hygromycin resistance for selection in *P. indica* and ampicillin resistance for selection in *E. coli.*

***U. maydis* transformation vectors:**

p123 - Vector possesses an eGFP gene which is fused to the otef promoter and nos terminator. For selection in *U. maydis* vector contains the carboxin resistance gene and for selection in *E. coli* an ampicillin resistance gene. Vector was kindly provided by Dr. G. Döhlemann, MPI Marburg, Germany, (Aichinger et al., 2003).

p123_10496 - The eGFP sequence (752 bp) from vector p123 was cut out with *BamH*I and *Not*I (NEB) and exchanged with the full length of PIIN_10496 (1065 bp). For selection in *U. maydis* vector contains the carboxin resistance gene and for selection in *E. coli* an ampicillin resistance gene.

p123_3173 - The eGFP sequence (752 bp) from vector p123 was cut out with *BamH*I and *Not*I (NEB) and exchanged with the full length of PIIN_03173 (1014 bp). For selection in *U. maydis* vector contains the carboxin resistance gene and for selection in *E. coli* an ampicillin resistance gene.

4.2. Bacterial, fungal and plant material

4.2.1. *Escherichia coli*

In this study two chemically competent *Escherichia coli* strains were used:

Organism	Strain	Source
Escherichia coli	DH5α	Invitrogen
Escherichia coli	K-12 Top10	Invitrogen

4.2.2. *Rhizobium radiobacter*

P. indica associated bacterial strain was tested during this study:

Organism	Strain	Source
Rhizobium radiobacter	PABac-DSM	(Sharma et al., 2008)

4.2.3. Sebacinales

Organism	Strain	Source
Piriformospora indica	DSM11827	Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany)
Piriformospora williamsii	DAR29830	Dr. Karl-Heinz Rexer (University of Marburg, Germany).
Sebacina vermifera	MAFF305830	National Institute of Agrobiological Sciences, Tsukuba, Japan
Sebacina vermifera	MAFF305842	National Institute of Agrobiological Sciences, Tsukuba, Japan

Piriformospora indica transformant strains generated during this study:

Strain	Description	Resistance cassette
Bshhn-pTEF	pTEF::HYG-tnos	Hygromycin
pvv26	RB-pTEF::HYG-tnos-LB	Hygromycin
Empty2, Empy4	Independent transformants carrying vector pPiRNAi used as empty vector control for RNAi studies	Hygromycin
Tam1_1, Tam2_1, Tam1_2, Tam2_2 and Tam3_1	Independent transformants with down regulated *piTam*1 gene (PIIN_07534)	Hygromycin
TP1, TP2, TP3…TP10	Independent transformants with overexpressed oGFP under promoter of *piTam*1 gene	Hygromycin
TGTh	pTEF::GFP-tCaMV 35S and pTEF::HYG-tnos	Hygromycin
TGFPh	pTEF::uGFP::HYG-tnos	Hygromycin
ToGFP	pTEF::oGFP::HYG-tnos	Hygromycin
MZGFP	pTEF::uGFP:piTEFterm and pTEF::HYG-tnos	Hygromycin
GoGFP	pGPD::oGFP-tnos and pTEF::HYG-tnos	Hygromycin

4.2.4. *Ustilago maydis*

For overexpression studies *U. maydis* solopathogenic strain was used:

Organism	Strain	Source
Ustilago maydis	SG200	(Kämper et al., 2006)

U. maydis overexpression strains generated during this study:

Strain	Description	Resistance cassette
123_GFP_2	otef::PIIN_03173-tnos	Carboxin
PIIN_03173_3, PIIN_03173_5	Independent transformants carrying otef::PIIN_03173-tnos expression cassette	Carboxin
PIIN_10496_3, PIIN_10496_4	Independent transformants carrying otef::PIIN_10496-tnos expression cassette	Carboxin
umTGTh	pTEF::GFP-tCaMV 35S and pTEF::HYG-tnos	Hygromycin
umTGFPh	pTEF::uGFP::HYG-tnos	Hygromycin
umToGFP	pTEF::oGFP::HYG-tnos	Hygromycin
umMZGFP	pTEF::uGFP:piTEFterm and pTEF::HYG-tnos	Hygromycin
umGoGFP	pGPD::oGFP-tnos and pTEF::HYG-tnos	Hygromycin

4.2.5. Plant species

Plant – microbe interaction studies and auxin activity analyses were performed using the following plants:

Organism	Strain	Source
Barley	*Hordeum vulgare* L. cv. Golden promise	Prof. Diter von Wettstein (Washington State University)
Tomato	*Solanum lycopersicum* L. cv. Hildares	HILD Samen GmbH (Marbach/Neckar, Germany)
Maize	*Zea mays L.* cv. Early *Golden Bantam*	Olds Seed Company, (Madison, Wisconsin, USA)

4.2.6. Media

All buffers and media were autoclaved 5-20 minutes at 121°C. In case of heat instability of ingredients, solutions were filter sterilized using Rotilabo® -syringe filters (pore size 0.45 µm or 0.22 µm; Roth; Karlsruhe).

4.2.6.1. *E. coli* cultivation

Bacteria were propagated overnight in dYT-medium (Sambrook et al., 1989) supplemented with 100 µg/ml ampicillin or 50 µg/ml kanamycin at 37°C and 200 rpm shaking for liquid cultures. For longterm storage glycerol stocks were prepared by mixing overnight bacteria culture with 80% sterile glycerol (1:1) and stored at -80°C.

dYT - medium
16 g/l tryptone
10 g/l yeast extract
5 g/l NaCl
13 g/l agar

4.2.6.2. Cultivation of *P. indica* and other Sebacinales

Selected species of Sebacinales possess the ability to grow axenically on many different synthetic media. For standard cultivation, modified complete medium (CM, Pham et al., 2004) was chosen. Cultures were propagated in 25-120 ml CM in 50-500 ml Erlenmeyer flasks at 28°C with 130 rpm shaking or on CM agar plates. *P. williamsii and S. vermifera* (MAFF305842) were grown on CM at 25°C. For cultivation of *S. vermifera* (MAFF305830) MYP medium was used at 25°C. Additionally, some other media (PDA, PDYA, PDYAS, MAE) were tested for *P. indica* cultivation. For influence of carbon source on the biosynthesis of IAA by *P. indica*, fungus was cultivated on minimal medium (MM) supplemented with different concentration of tryptophan and either with 2% glucose or with 2% arabinose. For longterm storage glycerol stock was prepared by mixing spores and mycelium pieces from 3-to 4-week-old culture plates, washed with 0.002% Tween water, with 80% sterile glycerol (1:1) and stored at -80°C.

CM - medium

50 ml/l 20 x salt solution*

20 g/l glucose

2 g/l peptone

1 g/l yeast extract

1 g/l casamino acids

1 ml/l 1000 x microelements**

15 g/l agar

20 x salt solution *

120 g/l $NaNO_3$

10.4 g/l KCl

10.4 g/l $MgSO_4x7\ H_2O$

30.4 g/l KH_2PO_4

1000 x Microelements **

6 g/l $MnCL_2x4H_2O$

1.5 g/l H_3BO_3

2.65 g/l $ZnSO_4x7H_2O$

750 mg/l KI

2.4 mg/l $Na_2MO_4x2H_2O$

130 mg/l $CuSO_4x5H_2O$

MYP - medium

7 g/l malt extract

1 g/l peptone

0.5 g/l yeast extract

12 g/l agar

Minimal medium (MM)

700 ml dd H_2O

20 g agar

autoclaved 5 min 121°C

100 ml 20 mM NH_4CL

100 ml 10x YNB (Yeast Base w/o amino acids and ammonium sulfate (Difco); filter sterilized)
100 ml 20% glucose

MAE - medium
25 g/l malt extract
15 g/l agar

PDA medium
39/l potato dextrose agar

PDYA - medium
39 g/l potato dextrose agar
25 g/l yeast extract
15 g/l agar

PDYAS - medium
19.5 g/l potato dextrose agar
1.5 g/l yeast extract
2 g/l KH_2PO_4
0.5 g/l $MgSO_4x7H_2O$
102.7 g/l sucrose (0.3 M)
15 g/l agar

4.2.6.3. *U. maydis* cultivation

U. maydis culture was prepared in Yeps light medium and grown in culture-tube at 28°C with 200 rpm shaking or on PDA agar plates. For long storage glycerol stocks were prepared by mixing overnight liquid culture with 80% sterile glycerol (1:1) and stored at -80°C.

Yeps light - medium
10 g/l yeast extract
10 g/l peptone
10 g/l sucrose

4.2.6.4. Plant cultures

Barley seeds were surface sterilized with 70% ethanol for 1 minute, 12% sodium hypochlorite for 1.5 hours and washed with sterile distilled water for 3 hours. Sterilized seeds were germinated in the dark for 3 days on sterile wet filter paper, at room temperature. For colonization studies the germlings were inoculated with *P. indica* chlamydospores as describe in chapter 4.3.4. Four germlings per treatment were grown on 100 ml 1/10 PNM in one sterile jar (Weck) in a Conviron growth chamber (Light period: 16/8 hours, Light intensity: approx. 108 µmol/m^2s, temperature: 22/18°C). For auxin activity test, tomato seeds were surface sterilized with 70% ethanol for 1 minute, 3% sodium hypochlorite for 15 minutes and washed with sterile distilled water for 45 minutes. Seeds were germinated 9 days on ½ MS medium with a day/night cycle of 16/8 hours (light intensity: approx. 47 µmol/m^2s) and 24°C permanent temperature. For *U. maydis* pathogenicity test, maize kernels were germinated in soil and grown in a greenhouse with a light period of 16/8 hours at 28°C/20°C.

1/10 PNM - medium
1 ml/l 500 mM KNO_3
1 ml/l from stock 5g /100ml KH_2PO_4
1 ml/l from stock 2.5g/100ml K_2HPO_4
1 ml/l 2 M $MgSO_4xH_2O$
1 ml /l 200 mM $Ca(NO_3)_2$
2.5 ml/l Fe-EDTA*
1 ml/l from stock 2.5g/100ml NaCl
4 g/l Gelrite
pH was adjusted to 5.6

Fe-EDTA*
2.5 g $FeSO_4x7\ H_2O$
3.6 g Na_2EDTA
400 ml water
Shortly boiled and cooled down, afterwards filled up to 1 l with dd H_2O

MS – medium

4.3 g/l MS including vitamins

15 g/l sucrose

8 g/l agar

4.3. Standard microbiological and biochemical methods

4.3.1. Heat shock transformation of *E. coli*

Preparation of competent cells and chemical transformation of *E. coli* succeeded according to a modified protocol of Cohen et al. (1972). 1 ml from an overnight preculture of *E. coli* cells was diluted to $OD_{600}\approx0.5$ with 100 ml LB medium supplemented with 10 mM $MgCl_2$ and 10 mM $MgSO_4$ and grown overnight at 37°C with 200 rpm shaking. The culture was centrifuged down at 4°C for 15 min at 3000 rpm (Sorvall RC 5B Plus). The supernatant was discarded and cells were resuspended in 33 ml of pre-chilled RF1 solution and incubated for 30-60 min at 4°C. The suspension was centrifuged at 4°C for 15 min at 3000 rpm and the supernatant was discarded. *E. coli* cells were resuspended in 5 ml of pre-chilled RF2 solution and incubated for 15 min on ice. Finally, 30 µl aliquots of competent cell suspension in sterile 1.5ml-Eppendorf microcentrifuge tubes were stored at - 80°C. In order to transform *E. coli*, concentrated aliquots were thawed on ice for 2 min. Before use, 270 µl of RF2 solution were added to each 30 µl aliquot. For each transformation, 50 µl competent cells were mixed with 2 µl ligation reaction, gently mixed and incubated on ice for 15-30 min. *E. coli* cells were then heat shocked at 42°C for 45 seconds and immediately cooled on ice for 2 minutes. For the recovery of the cells, 500 µl dYT medium without antibiotic was added and then the cells were regenerated for 30 min at 37°C. Finally, 150 µl of *E. coli* cell suspension was plated on dYT agar supplemented with 100 µg/ml ampicillin (dYTamp) or 50 µg/ml kanamycin (dYTkan). The rest of the suspension was then shortly centrifuged down, resuspended in 150 µl, plated on a second appropriate dYT agar plate and incubated at 37°C overnight. Additionally, on solidified plates, a mixture of X-gal and IPTG can be spread for blue/white selection of recombinants.

RF1 solution
100 mM RbCl
50 mM $MnCl_2x4H_2O$
30 mM potassium acetate
10 mM $CaCl_2x2H_2O$
15% (v/v) glycerol
pH was adjusted to 5.8 (NaOH)
Filter sterilized

RF2 solution
10 mM MOPS
10 mM RbCl
75 mM $CaCl_2x2H_2O$
15% (v/v) glycerol
pH was adjusted to 5.8 (NaOH)
Filter sterilzed

LB - medium
10 g/l peptone
5 g/l yeast extract
5 g/l NaCl
15 g/l agar
pH was adjusted to 7

4.3.2. Blue/white selection of recombinants

An advantage of using TA cloning is the possibility to quickly identify positive clones due to the blue/white selection of recombinants. Bacteria which possess vectors with correctly inserted PCR product are unable to produce β-galactosidase because of an interruption in lacZ gene sequence. Therefore, in the presence of X-Gal, these bacteria remain white, whereas bacteria with functional lacZ gene are able to convert it into

insoluble indigo pigment and turn blue. The white colonies are then used for further confirmation by colony PCR.

4.3.3. *P. indica* chlamydospores collection

Chlamydospores from *P. indica* and other Sebacinales were collected from 3– to 4-week-old culture plates using sterile tween water (0.002% of TWEEN20). Mycelium was stroked with a Drigalski-spatula and afterwards scratched with a scalpel. Mycelium pieces were then filtered through miracloth funnel (Calbiochem). Chlamydospores were collected in a sterile falcon tube, centrifuged down for 7 minutes at 3500 rpm, washed three times with tween water and set to a final concentration of 500000 chlamydospores per ml.

4.3.4. *P. indica*-barley interaction studies

For the interaction studies of *P. indica* with barley plants, experiments were performed under sterile conditions using surface sterilized and germinated (as described in chapter 4.2.6.4) barley seeds. Three-day-old germlings were dipped for 30 minutes in a chlamydospores suspension (0.75 ml of 500 000 spores per ml per germling) and transferred into sterile jars with 1/10 PNM. Additionally 1 ml of chlamydospores suspension was added to the medium in the proximity of the roots. Plants were cultivated in a Conviron growth chamber (Light period: 16/8 hours, Light intensity: approx. 108 µmol/m^2s, temperature: 22/18°C). Tween water treated germlings were used as a control. Root samples were collected after 3, 5 and 14 dai and carefully washed in distilled water. Roots were cut into two parts, the differentiation zone (3 cm below the seed) and elongation and meristematic zones (the rest of the root including root tip) and immediately frozen in liquid nitrogen. For the colonization studies only the differentiation zone was used as the heaviest colonized parts of the root in order to avoid the defusing effect of the much longer non colonized part of the root. All experiments were prepared in 3 to 4 technical replicates and 2 to 3 independent biological repetitions. The growth promotion test was conducted by dipping the pregerminated germlings into 50 ml 0.9% NaCl solution with 5 g crushed fungal mycelium (mycelium from liquid culture was washed three times with 0.9% NaCl and crushed with 1 to 5 short pulses of 2 seconds each by a sterile blender) for 1 h or 0.9% NaCl mock treated. Barley was grown in a 2 : 1 mixture (v/v) of expanded clay

(Seramis, Masterfoods, Verden) and Oil-Dri (Damolin, Mettmann, Germany) for 4 weeks. Each treatment comprised twenty plants with one plant per pot placed randomly in a plant growth chamber with a cycle of 14 h light (60 mmol /m^2s photon flux density) and 10 h of darkness at 22 and 18˚C respectively, and 60% relative humidity. The plants were watered from below twice a week and fertilized every second week with 7 ml l-1 Compo-Fertilizer (Compo Austria, Vienna, N/P/K: 6/4/5).

4.3.5. *U. maydis*-maize infection

Pathogenicity assays were performed using a modified protocol described previously by Kämper et al. (2006). *U. maydis* solopathogenic strain SG200 and transformant strains were grown in Yeps light medium overnight at 28°C to an OD600≈0.7-0.8, centrifuged down, washed twice with double distilled water and finally resuspended in double distilled water to a final OD600 of 1.0. The suspension was used to inoculate 7-day-old maize seedlings. Plants were kept in the greenhouse with a day/night cycle of 16/8 hours at 28°C/20°C. Disease symptoms were scored according to severity 7 dai (Table 3). Experiments were repeated three times and each replicate comprised about 55 infected plants.

Table 3 Description of observed plant symptoms.

Plant symptoms	Description
No symptoms	The plant has shown no signs of infection
Chlorosis	Pale yellowish chlorotic changes were observed on infected leaves
Anthocyans	Red brownish changes were observed on infected leaves
Tumors < 1 mm	The size of the largest tumors observed on the plant were smaller than 1 mm
Tumors 1-5 mm	The size of the largest tumors observed on the plant was between 1 and 5 mm
Tumors > 5 mm	The size of the largest tumors observed on the plant were bigger than 5 mm
Heavy tumors	Very high tumor formation was observed on infected leaves
Dead plants	The plants have died because of infection

4.3.6. *P. indica* protoplast preparation

Collected *P. indica* chlamydospores (2.5 x 10^6 chlamydospores/ml) were inoculated in 250 ml CM in a 500 ml sterile Erlenmeyer flask (with “Schikane”) and cultivated for about 7 days at 28°C with 130 rpm permanent shaking. Afterwards, the culture was filtered through miracloth filter and the mycelium was washed with 0.9% NaCl.

Mycelium was crushed for 10 seconds in 60 ml fresh CM using a sterile blender (Microtron MB 550, Kinematica AG). 20 ml of crushed mycelium was inoculated in 130 ml CM in a sterile Erlenmeyer flask and regenerated for 3 days at 28°C by 130 rpm. In this study two lysing enzyme solutions were analyzed: lysing enzymes from *T. harzianum* (20 mg/ml in SMC buffer; Sigma) and Novozyme (2.5 mg/ml in SCS buffer; Novo Nordisc; Kopenhagen, Dänemark). Both were filter sterilized using Rotilabo® - syringe filters (pore size 0.22 µm) and used in a total volume of 10–20 ml for one protoplastation reaction. Young crushed mycelium (regenerated for 3 days), was collected through miracloth funnel, washed with 0.9% NaCl and added into pre-chilled enzyme solution. Protoplastation was performed for 45-90 minutes at 37°C with *T. harzianum* enzyme solution and for 20-30 minutes at RT with Novozyme solution. After an appropriate amount of time, protoplast formation was checked under light microscope (Objective: A-plan 20x; Axiostar, Zeiss). If the amount and size of protoplasts were sufficient the protoplastation mixture was filtered through miracloth and enzyme activity was stopped by changing the pH of the mixture by adding equivalent amount of ice cold STC buffer. Subsequently, protoplasts were centrifuged down for 10 minutes by 4000 rpm at 4°C and gently resuspended in 5 ml cold STC. The centrifugation step was repeated twice (5 min, 4000 rpm, 4°C) with resuspending of protoplasts in 1 ml of STC. Protoplasts were finally resuspended in 350 µl STC, counted in a counting chamber (Neubauer improved) and diluted or concentrated to the optimal amount of protoplasts for transformation: 10^7-10^9 protoplasts per ml.

STC
1.33M Sorbitol in TC
50 mM $CaCl_2x2H_2O$
10 mM TrisHCl pH 7.5

SMC
1.33 M sorbitol
50 mM $CaCl_2x2H_2O$
20 mM MES buffer pH 5.8

SCS
20 mM sodium citrate, pH 5.8
1 M sorbitol

4.3.7. *P. indica* PEG-mediated transformation

For *P. indica* PEG-mediated transformation, 8-10 µg of linearized and gel-purified vector (Wizard, Promega) was used. 70 µl of freshly prepared protoplasts (10^7-10^9 protoplasts per ml) were mixed with previously linearized and cleaned vector and 1 µl of heparin (15 mg/ml) and incubated on ice for 10 minutes. Afterwards, to the transformation mixture 500 µl of STC/40% PEG and 1 µl of enzyme previously used for vector linearizing was added and incubated for another 15 minutes. For the regeneration, two different media was checked – CM and MYP medium both supplemented with 0.3 M sucrose (Sigma). The bottom part of the plate (20 ml) was prepared ahead and contained 100 µg/ml final concentration of hygromycin B (Roth) and 1.2% bactoagar. The transformation mixture was then gently mixed with 5 ml of pre-cooled to 37-45°C top agar supplemented with 0.6% bactoagar without any antibiotic. The transformation mixture embedded in the top agar was poured on solid bottom agar, solidified and cultivated at 28°C. After two weeks, transformants were transferred onto new CM plates with 80 µg/ml hygromycin B. To verified possibility of using frozen protoplasts aliquots, protoplasts were resuspended in STC buffer and mixed with 80% sterile glycerol (1:1) and stored at -80°C.

4.3.8. *U. maydis* protoplast preparation and PEG-mediated transformation

The protocol for *U. maydis* protoplast preparation and PEG-mediated transformation was modified based on Schulz et al. (1990) and Gillissen et al. (1992). The fungus was cultivated overnight in 4 ml Yeps light liquid medium at 28°C with 200 rpm permanent shaking. This culture was then diluted 1:300 in 50 ml fresh Yeps light medium and cultivated at 28°C until the optical density OD600 gained ~0.8 and then centrifuged down by 3200 rpm, washed with 25 ml SCS buffer and redissolved in 2 ml SCS with 2.5 mg/ml Novozyme. After 5-15 minutes protoplast formation was checked under a microscope. If the ratio of cigar-shaped cells to oval protoplasts was 1:1, the protoplastation was stopped by adding 10 ml SCS and centrifugation for 10 minutes at 4°C at 2300 rpm. The pellet was gently washed 3 times with STCum and finally resuspended in 0.5 ml of cold STCum buffer. Prepared protoplasts were used immediately for transformation or stored at -80°C. For PEG-mediated transformation,

70 µl of protoplasts were mixed together with 10-15 µl DNA (1-5 µg) and 1 µl heparin (15 mg/ml) and incubated for 10 minutes on ice. Subsequently, 0.5 ml STCum/40%PEG was added to the transformation mixture and incubated on ice for 15 minutes. Finally, the transformation mixture was plated on regeneration agar (10 ml top agar on 10 ml bottom agar supplemented with 8 µg/ml carboxin or 200 µg/ml hygromycin) and incubated at 28°C for 3-6 days. Transformants were transferred on fresh PDA plates supplemented with 2 µg/ml carboxin or 100 µg/ml hygromycin B, depending on selection gene present on the introducing vector.

STCum	**Regeneration agar**
100 mM $CaCl_2x2H_2O$	10 g/l yeast extract
10 mM TrisHCl pH 7.5	20 g/l pepton
1 M sorbitol	20 g/l sucrose
	182.2 g/l sorbitol
	15 g/l agar

4.3.9. Feeding tests

In order to confirm indole production in Sebacinales strains, feeding tests were performed. Therefore, fungal chlamydospores were collected and inoculated in 50 ml of CM or minimal medium M9. After 7 days of chlamydospores germination, fungal indole production was induced by application of the auxin precursor tryptophan at a final concentration of 2.5 mM, or one of the following indole derivatives: TOL, IPA, IAD, IAM, IAN, TAM and ILA. Negative controls were prepared from fungal pre-cultures by removing mycelium through the miracloth filter and adding appropriate indole derivatives into the cell-free medium. The supernatant was collected after 3 days of cultivation in complete darkness at 28°C with 130 rpm shaking through the miracloth filter and immediately used for further analysis or stored at -20°C. Dry biomass (dried in an 85°C oven) was measured. Additionally, for gene expression analyses of genes putative involved in auxin production in *P. indica*, time course studies were performed. In this case, supernatant samples were collected into sterile falcon tubes for indole extraction and mycelium was collected in miracloth filter, washed with sterile 0.9% NaCl and stored at -80°C for RNA extraction. For the *U. maydis* TRP feeding test, an

overnight culture was diluted to an OD600≈0.1–0.2 and cultivated for 30 hours in 20 ml Yeps light medium supplemented with 2.5 mM or 5 mM filter sterilized tryptophan. Fungal cultures were separated from the supernatant using a low-speed centrifugation step at 3500 rpm for 10 minutes and cell-free supernatant was used for indoles extraction. *Rhizobium radiobacter* was pre-cultivated in LB medium overnight, diluted to OD600≈0.05 and then cultivated in 25 ml LB medium supplemented with 0.5 mM tryptophan or 0.5 mM ILA. The culture was centrifuged down for 30 minutes at 4000 rpm and afterwards cell-free supernatant was used for indole extraction.

Minimal medium M9

6 g/l Na_2HPO_4

3 g/l KH_2PO_4

0.5 g/l NaCl

1 g/l NH_4Cl

autoclaved 5 min 121°C

20% glucose

1 M $MgSO_4$

4.3.10. Indole derivatives extraction and TLC separation

P. indica is known to release auxin into the medium during culturing (Sirrenberg et al., 2007, Vadassery et al., 2008). Prior to indole derivatives extraction, a Salkowski test was prepared for verification of auxin presence in solution. Thus, 1 ml of cell-free supernatant from the feeding tests was mixed with 2 ml of Salkowski reagent and 50 µl of 10 mM orthophosphoric acid. The mixture was then mixed well and incubated in complete darkness for 25 minutes. OD530 was then checked using spectrophotometer (Ultrospec 3000 pro).

Salkowski reagent

1 ml 0.5 M $FeCl_3$ (1.35 g in 10 ml)

50 ml 35% $HClO_4$

The 10 ml of cell-free supernatant from the feeding test was used for extraction of indole derivatives. A 3- to 4- hours long ethyl acetate extraction (supernatant:organic solvent in ratio 2:1) was executed by shaking at 200 rpm in complete darkness. Supernatant from *U. maydis* feeding test experiments was adjusted with 1M HCl to pH 3 prior extraction. The organic phase (ethyl acetate phase) was collected in 2 ml Eppendorf tubes and evaporated in a Speedvac machine sc110 (Savant, Thermo scientific, Germany) by medium speed level with heating for 30 minutes. The dry pellet was dissolved in 60 µl ethyl acetate. 10 µl of the extracted indole derivatives were sent for LC-MS/MS analyses to Dr. Lars Voll (Friedrich-Alexander University, Erlangen-Nürnberg, Germany), while 4 µl were loaded on a TLC plate (TLC silica gel 60, MERCK). The chromatographic chamber was saturated with the running buffer (chloroform:methanol:water; 84:14:1) for 1 hour and the TLC plates were run for 1- to 1.5 h. The plates were dried for 5 minutes and developed by spraying a mixture of van Urk and Salkowski reagents in the proportion of 1:3 (Ehmann, 1977) and incubated at 90°C for up to 10 minutes. For the identification of indole derivatives, 10 µl from 100 mM stocks prepared from commercially available standards (Sigma) were mixed and loaded onto a silica gel plate as a marker. The retention factor (Rf), defined as the distance travelled by the compound divided by the distance travelled by the solvent, was calculated for each of the indole derivatives. In order to separate ILA from TAM, which runs together in the standard running buffer used, a mixture of 2-propanol and water (2:1) was additionally used. For clear separation, TLC plates were run for 3 h.

4.3.11. Auxin activity assays

Auxin like activity of indole derivatives from culture supernatant extracts was verified using a coleoptile elongation test and an assay for IAA-dependent root formation on cotyledon explants. Coleoptile fragments of 0.5 cm were obtained from 3-day-old barley germlings after surface sterilization. *P. indica* and control cultures were propagated in CM as described above for the feeding test. Experiments were done in Nunc IVF 4 Well Dishes (Thermo scientific, Germany). For each treatment 50 coleoptiles were submerged in 1 ml supernatant solution and the elongation effect was measured after 24 h incubation at room temperature in complete darkness. The experiments were repeated twice.

Cotyledon explants were obtained from 9-day-old germlings grown from surface sterilized tomato seeds. Cotyledon halves were placed upside down on ½ MS medium supplemented with 1.5% sucrose, 0.8% agar and either 0 µM, 0.01 µM, 0.1 µM, 1 µM, 10 µM or 100 µM IAA or ILA. Additionally, 500 µM ILA and supernatant extract from a 96 h tryptophan-feeding test of *P. indica* and *S. vermifera* (MAFF305830) were used. The amount of root formation was measured after 12 days.

4.3.12. Dose-response growth assays

Effect of exogenously applied indole derivatives on fungal growth was evaluated by measuring the dry biomass from liquid culture experiments or the fungal colony diameter grown on solid medium. Different concentrations of IAA or one of the appropriate indole derivatives were supplied into 50 ml liquid CM or 25 ml CM with 1.5% agar (2.5 mM TRP, 250 µM IAD and/or 1 µM, 10 µM, 100 µM IAA). Liquid cultures were started from chlamydospores, cultivated for 7 days and then inoculated with indoles. Dry biomass was measured one week after induction. Agar plugs 5 mm in diameter were stamped out with a cork borer from the edge of 2 to 3-week-old plates and placed in the middle of prepared CM agar plates supplemented with appropriate concentration of appropriate indole derivatives. Colony diameter was measured after growth for 14 days at 28°C in complete darkness.

4.3.13. Protein extraction

About 300 mg of one week old *P. indica* mycelium from liquid culture was centrifuged down in a 2ml-Eppendorf tube at 4°C at 16000 rpm, subsequently washed three times with 1 x PBS and homogenized with mortar in 100 µl PBS/Tween20 (1 x PBS:Tween 1:5000). The mortar was washed out with additional 200 µl PBS/Tween20. This material was thoroughly vortexed and incubated on ice for 5-10 minutes. After a centrifugation step (10 minutes at 4°C at 14000 rpm) 10 µl supernatant was used for checking the protein concentration. Loading buffer was given to the rest of supernatant protein and denaturized for 5 min at 96°C. Protein concentration was checked with Bradford reagent (Roti-Quant, Roth). A 10 µl sample was mixed with 790 µl water and 200 µl Bradford reagent and measured in a Spectrophotometer (Ultrospec 3000 pro) at 530 nm. Amount of protein was determined based on a previously prepared BSA standard curve.

2 x Protein loading dye
2.5 ml 0.5 M Tris-HCl, pH 6.8
2 ml Glycerol
4 ml 10% (w/v) SDS
0.5 ml 0.1% (w/v) Bromophenol Blue
In total volume 10 ml
2.5 to 5.0%. β-mercaptoethanol was added before denaturation step to reduce intra or intermolecular disulfide bonds

5 x PBS
0.29 M Na_2HPO_4
0.085 M NaH_2PO_4
0.34 M NaCl
pH was adjusted to 7.4 (0.1 NaOH)

4.3.14. Polyacrylamide gel electrophoresis and Western blot

Protein production in obtained transformants was proved by Western blot analyses. Therefore, 20 mg of extracted total protein from *P. indica* liquid culture was mixed with protein loading dye and denatured for 5 minutes at 96°C, immediately cooled on ice for 2 minutes, shortly centrifuged and loaded on a polyacrylamide gel. Electrophoresis was run in SDS running buffer for 1.5 h with ~300 V; 20 mA per small gel. Afterwards proteins were transferred onto nitrocellulose membrane (Amersham Hybond ECL Nitrocellulose Membrane, GE Healthcare) in Trans-Blot SD Electrophoretic Transfer Cell (Bio-rad) for semi dry blotting. The machine was moistened with 1x blotting buffer and the transfer apparatus was constructed as follows (from below): wet (20% methanol) Whatman paper, nitrocellulose membrane, polyacrylamide gel and finally Whatman paper. All air bubbles were removed and the blotting was performed for 70-90 minutes with 0.12 A (~15V). Next, the membrane was briefly washed in order to remove blotting buffer with 1xwash buffer (Fast Western Blot Kits, SuperSignal West Pico - Pierce; Thermoscientific) and 10 ml of primary antibody solution (0.5 µg/ml; Living Colors A.v. Monoclonal Antibody (JL-8); Clontech) was applied and the

membrane was incubated for 30 minutes at room temperature. From this step onward all further steps were executed with permanent shaking. Subsequently, horseradish peroxidase-conjugated secondary antibody solution (HRP reagent) was used for 10–15 minutes. The membrane was then washed twice in 20 ml 1x wash buffer for 5-10 minutes. Finally, the SuperSignal West Pico Working Solution (5 ml of luminol with 5 ml of stable peroxide solution) was poured onto the membrane and incubated for 1- to 5 minutes at RT. After removing the last solution, the membrane was placed in a plastic wrap, exposed in a Roentgen cassette to the film (CEA RP New medical X-ray screen film blue sensitive) for 30 sec to 30 min and then the film was developed in a Fuji medical Film processor FPM-100A.

10% Separation gel (for 2 gels)	**5% Stacking gel (for 2 gels)**
4 ml dd water	2.25 ml dd water
2.5 ml 1.5M Tris-HCL (pH 8.8)	1 ml 0.5M Tris-HCl ph 6.8
3.32 ml polyacrylamide (30%)	666 µl polyacrylamide (40%)
100 µl 10%SDS	40 µl SDS 10%
100 µl APS (Ammonium persulfate, 10%)	40 µl APS
10 µl TEMED	4 µl TEMED

SDS running buffer	**1x blotting buffer**
25 mM Tris-HCl, pH 8.3	25 mM Tris-HCl, pH 10.4
192 mM glycerine	192 mM glycerine
4 mM SDS	15% (v/v) methanol

4.4. Standard molecular methods

4.4.1. DNA isolation

Fungal DNA was isolated from 10-day-old liquid culture. 100-200 mg of material, frozen at -80°C and ground in liquid nitrogen, was mixed with 1 ml of extraction buffer and incubated for 10 minutes at room temperature with permanent shaking. After the addition of 1 ml CIA (chloroform:isoamylalkohol, 24:1) and another 5 min of incubation, samples were centrifuged down for 20 min at 13000 rpm at room temperature. The aqueous phase was transferred in a new 2 ml-Eppendorf-tube and 0.2

reaction volumes of 100% EtOH were carefully added in order to precipitate polysaccharides. After 5 minutes of incubation with permanent shaking, 1 reaction volume of CIA was added and incubated for another 5 minutes followed by 20 minutes of centrifuging at 13000 rpm. The aqueous phase was again transferred into a new 2 ml-Eppendorf-tube and DNA was precipitated with 1 volume of isopropanol overnight at 4°C. Samples were centrifuged down for 30 minutes at 13000 rpm. The pellet was washed with 900 µl cold 70% EtOH. Ethanol was removed and the pellet was resuspended in 30-50 µl double distilled water. RNA was removed by addition of 1 µl of RNase A (10 mg/ml pH 7.4) and incubation at 37°C for 30-60 minutes. DNA quality and quantity was checked on 1% agarose gel and by NanoDrop-1000 Spectrophotometer.

Extraction buffer
100 mM Tris-HCl (pH 7.5)
50 mM EDTA (pH 8)
1.5 M NaCl
2% CTAB
0.05% ß-mercaptoethanol (added shortly before use)

1 M Tris-HCl pH 7.5	
88.8 g/l Tris HCl	(563 mM)
53 g/l Tris-Base	(437 mM)
pH was adjusted to 7.5	

0.5 M EDTA pH 8
182.1 g/l $Na_2EDTAx2H_2O$
In order to adjust the pH to pH 8 15 g NaOH pellets and afterwards 5 M NaOH solution was used.

DNA from *P. indica*-barley interaction studies was extracted using modified Doyle&Doyle protocol (1987). Around 200 mg of material, frozen (-80°C) and ground in liquid nitrogen, was mixed with 700 µl of prewarmed (65°C) D&D extraction buffer,

immediately vortexed and incubated for 30 minutes at 65°C with shaking 200 rpm. Afterwards samples were mixed with 700 µl CIA (chloroform:isoamylalkohol; 24:1) and mixed for 5 minutes at room temperature. After centrifugation step (20 min at 13000 rpm at 4°) the aqueous phase was transferred into a new 2ml-Eppendorf-tube and mixed with another 600 µl of CIA for 5 minutes followed by 10 minutes centrifugation (13000 rpm at 4°C). The aqueous phase was transferred into a new 2ml-Eppendorf-tube and mixed with 50 µl 10 M NH_4OAc and 60 µl 3 M NaOAc (pH 5.5) for 5 minutes. DNA was precipitated with 500 µl isopropanol overnight at 4°C after 5 minutes of incubation with shaking. The next day, samples were centrifuged down for 30 minutes at 4°C at 13000 rpm. The DNA pellet was washed with 500 µl 70%EtOH/10 mM NH_4OAc for 10 minutes. After removal of ethanol solution, the pellet was dried and then resuspended in 50 µl double distilled water. RNA was removed by the addition of 1 µl of RNase A (10 mg/ml pH 7.4) and incubation at 37°C for 30-60 minutes. DNA quality and quantity was confirmed on 1% agarose gel and by NanoDrop-1000 Spectrophotometer. Relative fungal DNA amount was calculated after qPCR analyses using the $^{2-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

D&D extraction buffer
100 mM Tris-HCl (pH 8)
20 mM EDTA (pH 8)
1.4 M NaCl
2% CTAB
1% $Na_2S_2O_5$
0.2% ß-mercaptoethanol; (added shortly before use)

3M NaOAc pH 5.5
408.1 g NaOAcx3H_2O in 600-800ml H_2O,
pH was adjust with acetic acid

4.4.2. Agarose gel electrophoresis

DNA or RNA was separated using agarose gel electrophoresis. Agarose concentration was dependent on the size of separated fragments and varied between 0.8% and 2%. The appropriate amount of agarose (Biozym) was dissolved in 1 x TAE or 0.5 x TBE buffer by cooking. After agarose solution was cooled down to 60°C, it was subsequently mixed with ethidium bromide (0.5 µg/ml; Roth) and poured in appropriate gel form. Separation took place in an electrophoresis chamber filled with 1 x TAE or 0.5 x TBE buffer and connected to a power source. DNA and RNA molecules are negatively charged in a neutral and alkaline environment therefore in an electric field they move towards the anode. DNA and RNA samples mixed with appropriate loading dye were loaded on the solidified gel and separated for 30-40 minutes by 120 V and 10 minutes by 135 V, respectively. DNA or RNA bands were observed under UV light and pictures were taken using UV solo TS imaging system (Biometra).

5 x TBE buffer
440 mM Tris-HCl
440 mM H_3BO_3
10 mM EDTA, pH 8

50 x TAE buffer
2 M Tris-HCl
2 M acetic acid
50 mM EDTA, pH 8

6 x DNA loading dye
40% (w/v) sucrose
0.25% (w/v) Bromphenolblue
to final volume 100 ml dd H_2O

RNA loading dye
50% (w/v) sucrose
0.25% (w/v) Bromphenolblue
0.25% (w/v) Xylencyanol FF
in 1x MOPS-Buffer

4.4.3. Polymerase chain reaction (PCR)

DNA fragments were amplified in order to carry out or confirm cloning or transformation steps. Thus, polymerase chain reaction (PCR) was performed in T-professional Basic Thermocycler (Biometra). Depending on application different polymerases were used. Standard PCR reactions were prepared using Taq polymerase

(Fermentas). For ligations, inserts were amplified using Pfu polymerase (Promega). Reaction mixtures and programs were performed as described in tables below. Additionally, to identify piGPD promoter sequence, inverse PCR (iPCR) was used after Triglia et al. (2000). 2 µg of *P. indica* genomic DNA was digested with the restriction enzymes *Sac*I, *Xba*I or *Xho*I (Fermentas) overnight at 37°C. After self-ligation prepared with T4 ligase (Promega), 400 ng the mixture was used as a template for iPCR with outer primers pGPDf/pGPDr followed by a nested PCR with the inner primers PGPD383f/PGPD383r. Obtained PCR product was then cloned into pGEMT-Easy and confirmed by sequencing. Afterwards, new primers were designed (GPD383f, PGPDHr, GPDf, PGPDH180r) on the fragment obtained and a second iPCR was performed after previous digestion with *Hind*III.

Standard PCR program			
5 min	95°C	initial denaturation	
30 sec	95°C	denaturation	
30 sec	55°- 65°C	annealing	X 29-34
30 – 90 sec	72°C	elongation	cycles
5 min	72°C	final elongation	

Standard PCR reaction

10-100 ng genomic DNA/cDNA

2.5 µl 10X Taq buffer with $(NH_4)_2SO_4$

2.5 µl 2.5 mM dNTPs (Fermentas)

2 µl $MgCl_2$ (Fermentas)

0.6 µl 10 pM primer forward (MWG)

0.6 µl 10 pM primer reverse (MWG)

0.5 µl Taq-polymerase (Fermentas)

dd water into 25 µl total volume

Standard PCR program for Pfu-polymerase			
3 min	95°C	initial denaturation	
30 sec	95°C	denaturation	
30 sec	Annealing temp. from PCR with Taq pol. – 5°C	annealing	X 29-34
2 – 4 min	72°C	elongation	cycles
10 min	72°C	final elongation	

PCR reaction with Pfu-Polymerase
10-100 ng genomic DNA/cDNA
5 µl 10X Pfu buffer with (Promega)
5 µl 2.5 mM dNTPs (Fermentas)
1.2 µl 10 pM primer forward (MWG)
1.2 µl 10 pM primer reverse (MWG)
0.5 µl Pfu-polymerase (Promega)
dd water into 50 µl total volume

4.4.4. TA cloning of PCR products

One of the easiest and fastest methods for insertion of PCR products into bacterial plasmid backbone is TA cloning. Vectors pGEM®-T Easy (Promega) and pCRII-TOPO (Invitrogen) are delivered in linearized form with a single 3´-terminal thymidine at both ends. The T-overhangs are ligated by ligase activity of T4 ligase or topoisomerase with compatible 3' deoxyadenosine overhangs for PCR products generated by Taq polymerase. In practice, standard ligation reactions were prepared as described in the tables below, ligated at room temperature for 1 hour and overnight at 4°C and finally heat-shock transformed into competent *E. coli* cells and plated on dYTamp ot dYTkan plates with X-Gal/IPTG (40 µl of 40 mg/ml X-gal and 40 µl of 100 mM IPTG).

pGEMTEasy cloning	pCRII -TOPO cloning
5 µl 2X Rapid Ligation Buffer	1µl salt solution
1 µl T4 DNA Ligase	1µl TOPO vector
1 µl pGEM®-T Easy Vector (50 ng)	4 µl PCR product
2 µl PCR product	6 µl
1 µl T4 DNA Ligase	
10 µl	

4.4.5. Southern blot

Stable transformation and integration of a transformed vector into the *P. indica* genome was confirmed by Southern blot. 10-40 µg isolated from 10-day-old liquid fungal culture DNA was digested overnight with an appropriate enzyme in a total volume of 100 µl. Afterwards, 1-2 µl of the digested DNA were run as control on an agarose gel. If digestion was sufficient, DNA was precipitated with a 1/10 reaction volume with 3 M NaOAc and a 2.5 reaction volume of 100% isopropanol for 10 min on ice. After a centrifugation step (10 min, 13000 rpm), the pellet was washed with 70% EtOH and subsequently dried under a laboratory hood. The DNA pellet was dissolved in 25 µl miliQ water, mixed with loading dye and loaded on 0.8% agarose gel (1 x TAE). Electrophoresis was run for 2.5-3 hours at 80 V. After photographing the gel (UV solo TS imaging system, Biometra), DNA was depurinated in 0.25N HCl for 15 minutes on a shaking platform and then denatured with 0.4 M NaOH for another 15 minutes on a shaking platform. Next, transferring apparatus was prepared (Fig. 46). Whatman paper (GE Healthcare), nylon membrane (Amersham Biosciences Hybond-N+, GE Healthcare) and paper towels were used. Transferring of DNA to membrane lasted 3.5 hours to overnight. Afterwards, membrane was crosslinked in UV Stratalinker 1800 (Stratagene) by an auto-crosslinking program [1200x100 µJoule]. Prepared membrane was pre-incubated in a hybridization tube with 20-30 ml of appropriate Southern

hybridization buffer for 0.5-2 hours at 65°C followed by hybridization overnight with DIG labelled or radioactively labelled probe.

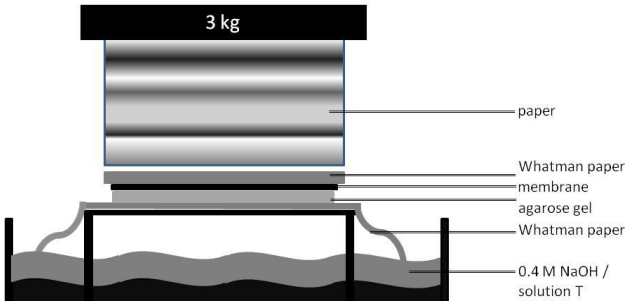

Fig. 46 Southern blot transferring apparatus

4.4.5.1. DIG labelling

In order to prepare a probe, 10 ng- to 3 µg DNA template (cleaned PCR product) in a final volume of 16 µl was denatured for 10 minutes at 95°C and quickly chilled on ice. Afterwards, 4 µl of DIG-High Prime (Roche) was added to the denatured DNA, mixed, centrifuged briefly and incubated for 1-20 hours at 37°C. Labelling was stopped by adding 2 µl 0.2 M EDTA pH 8 and incubating for 10 min at 65°C. After denaturation for 10 minutes at 100°C, the probe was added into 30-50 ml of pre-warmed hybridization buffer and used for hybridization at 65°C overnight. Next, the membrane was washed twice with Southern wash buffer for 20 minutes at 65°C and then 5 minutes in 30 ml of DIG-wash buffer at room temperature. The membrane was blocked in 30 ml of freshly prepared DIG II buffer for 30 minutes at room temperature. After a 0.5 hour incubation with antibody solution, membrane was washed twice with 30 ml DIG-wash buffer for 1 hour and then 15 minutes. The membrane was subsequently equilibrated for 5 min with 30 ml DIG III buffer at room temperature and then incubated with CDP-Star-solution for 5 minutes at room temperature. The membrane was put in a plastic bag (shrink-wrap), air bubbles were removed and the membrane was incubated for 15 minutes at 37°C in complete darkness. Under red light, the film (CEA RP New medical X-ray screen film blue sensitive) was placed on top of membrane in the Roentgen-cassette and incubated 30 seconds to 1 hour depending on bands intensity. The film was developed in Fuji medical Film processor FPM-100A.

Na-Phosphate buffer, 1 M, pH7
142 g/l Na_2HPO_4 138 g/l $NaH_2PO_4xH_2O$ Into 1 M Na_2HPO_4 solution 1 M $NaH_2PO_4xH_2O$ solution was added so long that the buffer gained pH 7.

CDP-Star-solution
100 µl CDP-Star (Roche) in 10 ml DIG III buffer (1:100)

Southern hybridization buffer
0.5 M Na-Phosphate buffer, pH7 7% SDS Stored at 37°C

Southern wash buffer
0.1 M Na-Phosphate pH7 1% SDS

DIG I buffer
0.1 M Maleic acid 0.15 M NaCl pH was adjusted to 7.5 with 5 M NaOH

DIG-wash buffer
0.3% (v/v) Tween-20 in DIG I buffer

Blocking reagent stock solution
10% blocking reagent (Roche) in DIG I (stored at 4°C)

Antibody solution
1 µl Anti-DIG-AP (Roche) in 10 ml DIG II buffer (1:10000)

DIG II buffer
Blocking reagent stock solution in DIG I buffer in 1:10

DIG III buffer
0.1 M NaCl
0.05 M $MgCl_2x6H_2O$
pH was adjusted to 9.5 with 1M Tris-HCl
pH 9.5

4.4.5.2. Radioactive labelling

For probe preparation ~100 ng cleaned PCR product diluted to final volume 25 µl with 1 x TE buffer was used. DNA was denatured for 5 minutes at 98°C and then cooled for 5 minutes on ice. For labelling, Amersham ready-to-go DNA Labelling Beads [$^{-32}$P] dCTP dissolved in 20 µl 1 x TE buffer were used. Denatured DNA was loaded on column, mixed with 5 µl of α-dCTP-^{32}P (=50 µCi) and incubated for 30–60 minutes at water bath (37°C). 50 µl of radioactivity sample was cleaned with previously prepared Illustra microspin G-25 columns (Amersham) by centrifugation for 2 minute at 735 rpm at 4°C. Denatured for 5 minutes at 95°C probe was mixed together with hybridization buffer and 1 ml of fresh boiled carrier DNA and was then applied to the hybridization tube instead of pre-hybridization buffer after overnight pre-hybridization at 65°C. After overnight hybridization, membrane was washed with solution A for 1 minute at 65°C. Solution was then discarded into radioactive waste and membrane was transferred into a clean tray and washed twice with 300 ml of solution A for 15 minutes at 65°C. After a 30 minute washing step with 300 ml of solution B, efficiency of radioactivity washing steps was checked with a Geiger counter. Clear membrane was dried with paper towels and put in a plastic bag (shrink-wrap). Air bubbles were removed and membrane was exposed to an X-ray film (XOMAT, Kodak) by incubation for 3- to 4 hours at room temperature in complete darkness in the Phosphor Imager box.

TE buffer
10 mM Tris pH 8
1 mM EDTA

Solution T
0.4 M NaOH

Pre-hybridization buffer
15 ml dd H_2O
6 ml HSB
3 ml Denhardts III
3 ml 10% SDS
3 ml fresh boiled Carrier DNA

Hybridization buffer
7 ml dd H_2O
3 ml 5 x HSB
1.5 ml Denhardts III
1.5 ml 10% SDS
1 ml fresh boiled Carrier DNA

5 x HSB
30.3 g/l PIPES diluted in 300 ml dd H_2O
pH was adjusted to 6.8
600 ml 5 M NaCl
40 ml 0.5 M EDTA Na_2x2H_2O

Denhardts III

4 g BSA – dissolve

20 g SDS – dissolve

4 g Ficoll-400 – dissolve

4 g PVP-360 – dissolve

10 g $Na_4P_2O_7x10H_2O$ – dissolve

Prepared in volume of 200 ml and added to 1500 ml of dd H_2O

Carrier DNA

125 mg DNA sodium salt from Salmon Testes

in 25 ml dd H2O

Shortly boiled and stored at -20°C

Solution A

100 ml 20 x SSC

100 ml 10% SDS

800 ml dd H_2O

Solution B

Solution A diluted with water 1:1

20 x SSC

3 M NaCl

0.3 M Trisodium citrate dihydrate

pH was adjusted to 7

4.4.6. RNA isolation and cDNA synthesis

Total RNA was extracted with TRIzol (Invitrogen, Karlsruhe). Thus, 100 mg of grounded frozen material was mixed with 1 ml TRIzol and vortexed directly. Afterwards, 200 µl of chloroform was added and samples were vortexed for 20 seconds. After 30 minutes centrifugation (4°C 13000 rpm), 500 µl of aqueous phase was

transferred into a new 2 ml-Eppendorf-tube and precipitated with 500 µl isopropanol for 1 hour at 4°C. The pellet obtained after centrifugation for 30 minutes at 4°C at 13000 rpm was washed with 1 ml of 75% EtOH (diluted in DEPC water) and vortexed briefly. Dried RNA pellets were redissolved in 40 µl RNase-free water at 65°C for 5 minutes. Amount and quality of extracted RNA was estimated by Nanodrop and on agarose gel. 1-2 µg RNA was used for DNase I digestion followed by cDNA synthesis. qPCR was performed from 10 ng cDNA/DNA mixed with the appropriate primers in 10 µl SYBRgreen Supermix (Bio-rad) using the following amplification protocol: initial denaturation for 10 min at 95°C, followed by 40 cycles with 30 s at 95°C, 30 s at 59°C, 30 s at 72°C and a melt curve analysis. Relative expression and its fold changes values were calculated using the $^{2-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

Standard real time qPCR reaction
4 µl cDNA / DNA (10 ng)
10 µl SYBR green mix
0.2 µl 10 pM forward primer
0.2 µl 10 pM reverse primer
0.4 µl 1µM FITC
Water into total volume 20 µl

DNAse I digestion (Fermentas)
2 µg RNA
2µl 10X reaction buffer with $MgCl_2$
2 µl DNase I, RNase-free (1U)
0.5 µl Ribolock RNase inhibitor (40U/µl)
dd water into total volume 20 µl
30 minutes at 37°C
1 µl 50 mM EDTA
10 minutes at 70°C

First Strand cDNA Synthesis (Fermentas)
10 µl RNA after DNaseI digestion
1 µl oligo $(dT)_{18}$ primer
1 µl random hexamer primer
5 min at 65°C
5 min 4°C

4 µl 5x reaction buffer

1 µl Ribolock RNase inhibitor (20 U/µl)

2 µl 10mM dNTPs

1 µl M-MuLV Reverse Transcriptase (20 U/µl)

5 min at 25°C

60 min at 45°C

5 min at 70°C -> 1 µg RNA diluted to 2.5 ng/µl

4.5. Microscopy

For chlamydospore and protoplast counting, an optical microscope (Objective: A-plan 20x; Axiostar, Zeiss) was used. GFP fluorescence was analyzed using a confocal laser scanning microscope TCS-SP5 (Leica, Bensheim). The green fluorescence was excited with an argon laser (30% strength) at 488 nm and detected at 505-560 nm.

Fluorescence intensity of the hyphae during the Tam promoter studies was measured using the corrected total cell fluorescence (CTCF) method (Gavet & Pines, 2010) in the software ImageJ (http://rsbweb.nih.gov/ij/). For GFP intensity measurements, the following formula was used:

The whole corrected hyphal cell signal = whole hyphal cell signal − (area for the selected cell x mean background)

The whole hyphal cell signal = sum of the intensity of the pixels for one area; mean background signal = mean signal per pixel for a region selected just beside the hyphae. For each measurement 10 to 20 regions from 2 to 3 independent pictures were used. All pictures were taken under the same conditions: Objective: HCX PL APO lambda blue 20.0 x 0.70 IMM UV; zoom: 3.3, photo multiplier tube PMT1 (HV): 906.1.

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Zurawska M., Delis M, Galazka J., Bartoszewski G., Niemirowicz-Szczytt K 2007: *Agrobacterium*-mediated transformation of cotyledon explants of pepper (*Capsicum annuum* L.). W: Niemirowicz-Szczytt K. (Ed.), Progress in Research on Capsicum & Eggplant. Warsaw University of Life Sciences Press, Poland, 355 – 364.

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